PATHOGENICITY OF VIBRIO ANGUILLARUM IN FISH

BY

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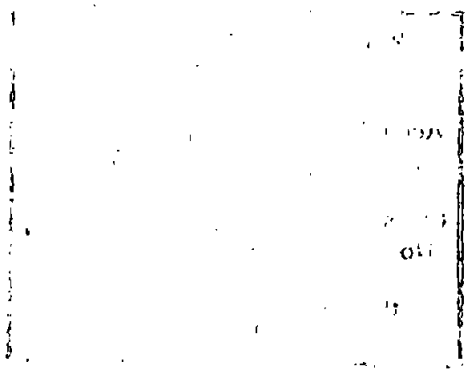
THESIS SUBMITTED TO THE COUNCIL FOR NATIONAL ACADEMIC AWARDS IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PLYMOUTH POLYTECHNIC, 1981



Abstract

This dissertation reports the first detailed study of the possible pathogenic mechanisms and virulence determinants of the fish pathogen V.anguillarum,carried out in association with histopathological studies using electron microscopy and electrocardiography In addition,possible infection route(s) were investigated and the effect of environmental variables on pathogenesis examined.

Pathogenicity was found to be species specific,with strains which were avirulent in eels being highly virulent in grey mullet. Following injection of virulent bacteria into eels,all tissues were rapidly colonised,death being characterised by severe haemorrhagic septicaemia.In contrast, avirulent strains were rapidly eliminated from the eel host to levels below detection.The heart was particularly affected during pathogenesis. Vibriosis was found to be temperature related, disease being retarded by low temperature.

Pathogenic properties investigated included production of enzymes and haemolysins and examined in vitro phagocytosis and growth rates. Membrane proteins were extracted by various techniques and separated using SDS-polyacrylamide electrophoresis thereby elucidating interstrain variation in protein profile, most strains containing a major outer membrane protein thought to be a porin. Ultrastructural studies revealed some bacteria to have up to three polar flagella per cell, with multiflagellate forms only being observed in virulent strains. Analysis of plasmid DNA revealed a partial correlation between possession of a 47 megadalton plasmid and colistin resistance.

Experimental vibriosis was characterised by deposition of haemosiderin in liver tissues,thought to be a poorly studied host defence mechanism, and large numbers of tissue bacteria surrounded by an electron lucent zone which was non-capsular in nature. Features of the disease included desquamation of the intestinal mucosa with excessive loss of ions into the gut lumen.

The most likly route of infection was thought to be via the gut, as osmoregulatory processes provided a direct means for waterborne V.anguillarum to enter the gut, to which this bacterium was found to be particularly well adapted. Gut traversal was thought to be the precursor to a possible latent infection in the kidney.

Declaration

I hereby declare that this thesis has been composed by myself,that it has not been accepted in any previous application for a higher degree,that the work of which it is a record has been performed by myself,and that all the sources of information have been specifically acknowledged.

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Acknowledgements

I would like to express my gratitude to the following people for their assistance in various ways during the course of this study :-

Dr C.B.Munn for supervision and permission to include results of plasmid analysis and biochemical test data, in this thesis.

Dr S.E.Egglestone for many helpful discussions throughout the research.

Dr D.Bucke for invaluable instruction in microscopy techniques.

Dr P.Owen for expert instruction and discussion in electrophoresis; and hospitality during my visit to Trinity College Dublin.

Drs P.Glynn and R.A.Matthews for helpful discussion and advice.

Dr A.M.Glauert for discussion concerning microbial ultrastructure.

Commander M.V.Ingram for specimens of naturally infected eels.

The Marine Biological Association for facilities to study the bacterial flora of coastal fish.

The technicians of the Biological Sciences department and Media Services.

Drs T.Håstein, J.S.Rohovec, A.J.Novotny, J.Larsen and H-Y.Chung for donating strains of *V.anguillarum*.

And finally, to Miss Marsha Rapson for typing.

This research was carried out while in receipt of a Science Research Council grant for which I make grateful acknowledgement.

Research was carried out under Home Office licence number :- SWI 2789.

Advanced studies

During the programme of research, advanced studies were carried out :-

i) Received instruction on protein separation techniques from Dr P. Owen, at Trinity College Dublin.

ii) Attended a 'Careers, Research and Advisory Course' at Stirling University, Scotland; sponsored by the Science Research Council.

In addition, during the research, visits were made to :-

iii) National Institute for Medical Research, Mill Hill, London.

iv) Unit of Aquatic Sciences, University of Stirling, Scotland.

v) A paper was presented at the Third COPRAQ-Session, Munich, October, 1979.

Publications

Part of the research has been published :-

Chart,H. & Munn,C.B. (1980) Experimental vibriosis in the eel (Anguilla anguilla). In: Ahne,W. (ed) Fish Diseases, Third COPRAQ-Session, Springer-Verlag, Berlin, Heidelberg & New York.

Contents

Abbreviations used

AB	Alcian Blue
BSA	Bovine Serum Albumen
^{o}C	Degrees centigrade
CIE	Crossed immunoelectrophoresis
cm	centimetre
C	Cytosine
dm	Decimetre
DNA	Deoxyribonuleic acid
DNase	Deoxyribonulease
ECG	Electrocardiography
EDTA	Ethylenediaminetetracetic acid
EDTA.Na_2	Ethylenediaminetetracetic acid-di-sodium salt
Fig	Figure
mEq	milliequivalent
Formol-PBS	10%^{v}/v formalin in PBS
g	Standard acceleration of gravity
g	Gram
µg	micro gram (10^{-6})
G	Guanine
HBSS	Hank's balanced salt solution
hr	Hour
HU	Haemolytic unit
im	Intramuscular(injection)
ip	Intraperitoneal(injection)
iv	Intravascular(injection)

Kg	Kilogram
LD-50	Lethal Dose-50
LS	Longitudinal section
M	Molar
mM	millimolar
μM	micromolar
MBA	Marine Biological Association of the United Kingdom
MIC	Minimum inhibitory concentration
min	minute
nd	not determined
nm	nanometre (10^{-9})
0/129	2,4-diamino-6,7-diisopropylpteridine (vibriostatic compound)
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline
R-factors	Transferable resistance factors
RDC	Rapid decalcifier
sec	second
SEM	Scanning Electron Microscope
sc	Subcutaneous(injection)
TEM	Transmissiom Electron Microscope
Tris	Tris(hydroxymethyl)methylamine
TS	Transverse section
TSA	Tryptone soy agar
TSB	Tryptone soy broth
UIBC	Unsaturated Iron Binding Capacity
V	Volt
mV	milliVolt
μV	microVolt
v/v	volume:volume
wt	weight
w/v	weight:volume

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CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1.0 INTRODUCTION

Aquaculture has been carried out for several thousand years as a means of producing food through inputs of labour and energy, to improve the yield of aquatic organisms by deliberate manipulation of the rates of growth, mortality and reproduction. Thus, instead of adapting to the natural constraints of production and yield as in capture fisheries, deliberate attempts are made to remove these constraints in aquaculture.

Early culture systems tended to involve particular trophic levels in the food chain such that animal sewage used to fertilize aquatic plants subsequently provided food for herbivorous fish species. Recently, aquaculture has become commercialised with a tendency towards farming species which provide the best financial gain as opposed to providing a necessary protein source.

In 1975, world aquaculture production was estimated at 6M tons composed of fin fish (66%), molluscs (16.2%), crustacea (0.3%) and seaweeds (17.5%). Fish culture, constituting the largest proportion of aquacultural production, has been carried out on a global scale, with Middle-Eastern and Asian countries primarily concerned with the rearing of food fish, while western countries, also culture species for sport and aquarists.

Although precautions are taken to isolate stocks from external interference, cultured fish are susceptible to diseases caused by microbial pathogens. With farming practices including monoculture, usually of a similar age group and under unnaturally high stocking densities, fish are susceptible to transmissible diseases which can reach catastrophic proportions. Of the bacterial diseases to have a significant effect on fish culture, vibriosis and furunculosis are considered the most important in marine and fresh water respectively.

The economics of fish culture have initiated interest into means of disease prevention and have provided support for research into vaccination and chemotherapy.

Vibriosis, caused by Vibrio anguillarum, is a disease principally of marine fish, characterised by haemorrhagic septicaemia. V. anguillarum has been reported world-wide and is apparently able to infect all teleosts (Anderson & Conroy, 1970), having most dramatic effect on the salmon industries of North America and the eel and salmonid culture of Japan. The paucity of information on pathogenesis of vibriosis in non-salmonid species has limited the development of control methods, and this thesis reports the research carried out on Vibrio anguillarum and its interaction with fish, and relating these aspects to a detailed study of pathogenesis. Experimentation was carried out predominantly using the freshwater eel (Anguilla anguilla) as this economically important species has been shown to be susceptible to vibriosis and is easily maintained in captivity.

The overall aim of the research was to examine the pathogen-host relationship with particular emphasis on pathogenic mechanisms of V. anguillarum and possible virulence determinants.

The aims of the project were as follows:-

1) To establish experimental systems for the study of vibriosis, including techniques for maintenance and infecting of fish.
2) To investigate the pathology of vibriosis assisted by histopathology at light and electron microscopical levels, and ascertain the effects of environmental influences on pathogenesis.
3) To determine the pathogenicity of a number of strains of V. anguillarum and relate virulence to biochemical, ultrastructural and bacteriological properties, to elucidate possible virulence determinants.

1.2.0 LITERATURE REVIEW

This literature review deals predominantly with the published work concerning vibriosis in fish and the causative organism *Vibrio anguillarum*. However, where appropriate, other bacterial pathogens have been mentioned. To assist in comprehension of the literature, areas of research have been placed under key headings.

1.2.1 Taxonomy

Vibrio anguillarum is a member of the family *Vibrionaceae* (Veron, 1965). Members of the *Vibrionaceae* are rigid, gram-negative, rod-shaped bacteria with straight or slightly curved morphology. They are usually motile by polar flagella although some may produce lateral flagella under certain culture conditions. They are chemo-organotrophic, able to carry out both fermentation and respiration, oxidase positive, with several species producing butylene glycol from glucose. Some are proteolytic and/or produce indole. They are facultatively anaerobic and usually found in fresh or sea water; having a G + C content of their DNA ranging from 39-60 moles percent. The genus *Vibrio* is further characterised by its sensitivity to 2, 4 diamino-6,7-di-iso-propyl pteridine (O/129) and has a G + C content of 40-50 moles percent. *V. anguillarum* is differentiated from other members of this genus by culture temperature, NaCl requirement and its ability to produce disease in fish (Buchanan & Gibbons, 1975).

The organism was first mentioned by Canestrini in 1893 who named the organism responsible for an epizootic in eels: *Bacillus anguillarum*; later to be renamed *Vibrio anguillarum* by Bergman (1909). Strains of this organism isolated from outbreaks were initially thought to be different species: *V. piscium* (David, 1927), *V. ichthyodermis* (Zobell & Wells, 1934) and *V. piscium var japonicus*

(Hoshina, 1957). However, Hendrie et al. (1971) carried out extensive tests on these 'species' and proposed that the above isolates were the same species, Vibrio anguillarum Bergman.

Further taxonomic studies using DNA hybridisation have been carried out to clearly distinguish V. anguillarum from closely related species (Hanoka et al., 1969; Kiehn & Pacha, 1969; Anderson & Ordal, 1972; Staley & Colwell, 1973). Within the species V. anguillarum, biochemical investigations have differentiated available strains of this organism into biotypes. Nybelin (1935) was able to separate strains into two biotypes based on acid, but no gas production from sucrose and mannitol, and production of indole (type A) and lacking the ability to utilize sucrose and mannitol or produce indole (type B). Smith (1961) proposed a third biochemical type (type C) for those strains that ferment sucrose and mannitol but fail to produce indole. Håstein & Smith (1977) were able to separate their strains on the basis of arabinose utilization, revealing that isolates from farmed salmonids tended to be arabinose positive (group 1) while isolates from wild fish were generally arabinose negative (group 2).

Following recent serological studies on V. anguillarum strains (Schiewe, in press), it was suggested that V. anguillarum biotype 2 should be considered as a separate species : V. ordalii

1.2.2 The occurrence and history of vibriosis

Vibriosis (red pest or red boil disease; Bergman, 1909) is a serious and economically important disease which has been reported in several European countries including: Denmark, Germany, Italy and Sweden. During the years 1925-1927, a serious outbreak of 'red boil' disease was reported in Germany (Schaperclaus, 1927) with V. anguillarum being isolated from both eel and pike. In 1952 an outbreak of vibrio

disease was reported in Germany, producing serious mortality in plaice (Buckman, 1952) with an outbreak being reported in Danish coastal waters (Bagge & Bagge, 1956). Vibriosis was first reported in North America in 1953 by Rucker, Earp & Ordal (1953) following disease in salmon, while vibriosis was first reported in Canada in 1971 by Evelyn also following an outbreak in salmon.

The occurrence of vibriosis and the species affected is summarised by Anderson & Conroy (1970). This data has been updated by the addition of literature published on vibriosis since the publication of above (Table 1).

1.2.3 Entry of host by *V. anguillarum*

The means by which the bacterium enters the host fish remains to be clarified, however it appears that access to host tissues could be gained by either a vector, by lesions and abrasions or by active/passive penetration of the fish external surfaces.

Håstein and Bergsjo (1976) have shown that lesions in the skin of Atlantic salmon (*Salmo salar*) produced by the ectoparasitic copepod *Lepeophtherius salmonis* provided access to the tissues for *V. anguillarum* subsequently leading to vibriosis. Roberts *et al.* (1973) found *V. anguillarum* penetrated salmon via lesions produced by marker-tags. Griffin (1953) implicated leeches and fish lice (*Argulus* spp.) in outbreaks of infectious dropsy in carp caused by *Aeromonas liquefaciens*. Although invertebrate vectors appear to provide a means for potentially pathogenic bacteria traversing the fish skin, it seems unlikely that this route could account for the sudden high mortalities experienced in, for example, the North American salmonid culture.

It has been suggested that outbreaks of vibriosis may have been caused by populations of *V. anguillarum* harboured within the fish body

Table 1 The geographical distribution of fish from which Vibrio-associated diseases have been described

Geographic location	Fish species	Author
America	Crocker (Icropogon opercularis)	Conroy, 1970
Argentina	Smelt (Atherinops affiis)	
Canada	Herring (Clupea pallasi)	
USA	Killifish (Fundulus parvipinnis)	Cisar & Fryer, 1969
	Cod (Gadus macrocephalus)	Oppenheimer, 1962
	Goby (Gillichthys mirabilis)	Ross, Martin & Bressler, 1968
	Blenny (Hypsoblennius gilberti)	Rucker, 1959
	Pink salmon (Onchorhyncus gorbuscha)	Rucker, Earp & Ordal, 1953
	Chum salmon (O.keta)	ZoBell & Wells, 1934
	Chinook salmon (O.tshawytsha)	
	Rainbow trout (Salmo gairdneri)	
	Queenfish (Seriphus politus)	
	Chum salmon (O.keta)	Evelyn, 1971
	Chinook salmon (O.tshawytsha)	Cardwell & Smith, 1971 Didier, 1974
	Winter flounder (Pseudopleuronectes americanus)	Levin, Wolke & Gabrielli, 1972
	Cod (Gadus morhua)	Traxler & Li, 1972
	Freshwater tropical fish: (Lebistese) (Cyprinidae) (Cobitidae)	Hacking & Budd, 1971
Asia	Mandeli (Coilia dussumeri)	
India	Moonfish (Drepane punctata)	Almeida, de Salva & Freitas, 1968
	Ox-eye (Megalops cyprinoides)	
	Scat (Scatophagus argus)	
	Rabbitfish (Teuthis vermicularis)	
	Tilapia mossambica)	
	Pampano (Trachinotus ovatus)	
Japan	Puffer (Fugu rubripes)	
	Wrasse (Halichoeres pocilopterus)	
	Parrot bass (Oplegnathus faciatus)	Hoshina, 1956
	Wrasse (Pseudolabrus japonicus)	Kubota & Takakuwa, 1963
	Ayu (Plecoglossus altivelus)	Kusuda, 1966
	Rainbow trout (S.gairdneri)	Muroga & Egusa, 1967
	Yellow tail (Seriola sihama)	Jo, Ohnishi & Muroga, 1979
	Smelt (Siiago sihama)	
	Triggerfish (Stephanolepsis cirrhifer)	
	Horse mackerel (Trachurus japonicus)	Saito, Oturu, Furukawa, Kanda & Sato, 1964
	Rainbow trout (S.gairdneri)	
	Red sea bream (Chrysophrys major)	Hirano & Yone, 1971
	Eel (Anguilla japonica)	Miyagaki, Jo, Kubota & Egusa, 1977

Table 1 continued

Geographic location	Fish species	Author
Europe		
Austria	Carp (Cyprinus carpio)	David, 1927
	Pike (Esox lucius)	
Denmark	Eel (Anguilla anguilla)	Bagge & Bagge, 1956
	Cod (Gadus morhua)	Bruun & Heiberg, 1935
France	Plaice (Pleuronectes platessa)	Jacquet, Villette & Renarad, 1959
	Sand eel (Ammodytes tobianus)	
	Eel (Anguilla anguilla)	Lagarde & Chakroun, 1965
Germany	Eel (Anguilla vulgaris)	
	Pike (Esox lucius)	Buckman, 1952
	Roach (Leuciscus rutilus)	Mattheis, 1964
	Flounder (Pleuronectes flesus)	Schapperclaus, 1927, 1928, 1934
	Plaice (Pleuronectes platessa)	Wolter, 1960
Ireland	Atlantic salmon (Salmo salar)	Edwards, O'Kelly, Napier & Fletcher, 1960
Italy	Eel (Anguilla vulgaris)	Canestrini, 1893
	Rainbow trout (S.gairdneri)	Ghittino, Andruetto & Vigliani, 1972, 1975
Norway	Pike (Esox lucius)	Aser, 1925
	Rainbow trout (S.gairdneri)	Holt, 1970
	Saithe (Pollachius virens)	Egidius & Andersen, 1975
	Coalfish (Gadus virens)	Håstein & Holt, 1972
	Cod (Gadus morhua)	
	Dab (Limanda limanda)	
	Flounder (Pleuronectes flesus)	
	Plaice (Pleuronectes platessa)	
	Salmon (S.salar)	
Poland	Eel (Anguilla anguilla)	Kocylowski, 1963
Sweden	Eel (Anguilla anguilla)	
	Cod (Gadus morhua)	Bergman, 1909, 1912
	Wrasse (Labrus spp)	Ljungeberg, 1963
	Plaice (P.platessa)	Nybelin, 1935
	Turbot (Scophthalmus maximus)	
	Gurnard (Trigla spp)	
UK	Eel (Anguilla anguilla)	Chart, 1980 this thesis.
	Cod (Gadus morhua)	
	Roach (L.rutilus)	
	Dab (L.limanda)	Rushton, 1957
	Lemon sole (Pleuronectes microcephalus)	Smith, 1959, 1961
	Plaice (P.platessa)	Anderson & Conroy, 1970
	Brill (Rhombus maximus)	
	Sea trout (Salmo trutta)	
	Sand sole (Solea lascaris)	
	Dover sole (Solea vulgaris)	McCarthy, Stevenson & Roberts, 1974
	Rainbow trout (S.gairdneri)	
	Turbot (S.maximus)	Horne, Richards, Roberts & Smith, 1977
	Eel (A.anguilla)	MaCarthy, 1976

(Bullock et al., 1971; Wood, 1974; Håstein, 1974). Rødsaether et al. (1977) found that eels contracted vibriosis after being exposed to copper, even though these fish had been maintained in fresh water for eight months. Hirano and Yone (1971) found a similar situation when apparently healthy red sea bream (Chrysophrys major) contracted vibriosis after experiencing transportation stress.

Håstein (1974) has suggested that filter feeding invertebrates such as the tunicate Ciona intestinalis may act as a natural reservoir for water borne V. anguillarum. Grischrowsky (1973) reported an outbreak of vibriosis in the pacific oyster (Crassostrea gigas) which is also a filter feeder and could accumulate and concentrate water borne V. anguillarum. Fish, feeding on living or diseased individuals of these and related organisms, would receive large doses of pathogenic bacteria, perhaps leading to vibriosis or a latent infection. Outbreaks of vibriosis have been reported following the feeding of contaminated feedstuffs to farmed fish (Rucker, 1959; Ross et al., 1968; Ross, 1970). It would seem likely that V. anguillarum can therefore survive in the fish gut and subsequently invade the host tissues across the gut mucosa.

Whether this pathogen remains in the gut or enters the fish tissues by traversal of body surfaces is unclear. However, to remain in the fish as a latent form, V. anguillarum, would require mechanisms to avoid being disposed of by the host defence system.

Latency appears to have been poorly studied in fish, however several mammalian microbial pathogens have a latent existence in the host tissues. For an organism to remain in the host, it has to avoid being killed by host defence mechanisms; for example, Mycobacterium tuberculosis can persist in the host by remaining viable within macrophages. Lytic enzymes, held in lysosomes, are prevented from

fusing with the phagosome containing the M. tuberculosis (Vernon-Roberts, 1972), and consequently the bacteria can remain viable.

1.2.4 Gross manifestations and histopathology of vibriosis

The literature concerning the manifestations of vibriosis is extensive, and describes the disease in a range of fish species with most detailed work on salmonids. Although vibriosis has been reported in wild fish (Smith, 1961; Muroga & Egusa, 1967; Håstein & Holt, 1972; Levin et al., 1972; Traxler & Li, 1972), initial signs of the disease have been studied mainly in captive fish (Evelyn 1971; Novotny et al., 1975; Novotny, 1978).

Early signs of vibriosis are exhibited by individuals becoming dark and lethargic accompanied by loss of appetite (Bruun & Heiberg, 1932; Anderson & Conroy, 1970; Roberts & Shepherd, 1974; Harbell, 1976; Roberts, 1978). External pathological features include petechial haemorrhages of the skin (Smith, 1961; Bullock et al., 1971; Håstein & Holt, 1972), lesions and ulceration of musculature (Pacha & Kiehn, 1969; Håstein & Holt, 1972; Levin et al., 1972; Traxler & Li, 1972). The fins become congested and haemorrhaged (Smith, 1961; Anderson & Conroy, 1970) and exophthalmus may be present (Ross et al., 1968; Håstein & Holt, 1972; Hodgins et al., 1977; Horne et al., 1977; Richards, 1980).

Dissection of infected fish reveals internal haemorrhaging including swelling and liquefaction of spleen and kidney (Ross et al., 1968; Bullock et al., 1971; Roberts & Shepherd, 1974; Novotny et al., 1975; Harrell et al., 1976; Roberts, 1978). The liver appears congested, swollen and haemorrhaged (Bruun & Heiberg, 1932; Ross et al., 1968; Anderson & Conroy, 1970; Novotny et al., 1975; Harrell et al., 1976; Roberts, 1978); with heart and gills also being affected

(Horne et al., 1977; Roberts, 1978; Richards, 1980). The gut appears red and haemorrhaged with vasodilation of mesenteries (Bruun & Heiberg, 1932; Saito et al., 1964; Ross et al., 1968; Hodgins et al., 1977) and may contain a viscous exudate (Saito et al., 1964; Anderson & Conroy, 1970; Novotny et al., 1975; Harbell, 1976). Examination of the musculature reveals haemorrhages in the muscle myotomes (Muroga & Egusa, 1967; Harbell, 1976).

The histopathology of vibriosis has been studied in less detail, but does suggest certain common features in disease manifestations at the light microscope level. A general feature of the disease in winter flounder (Pseudopleuronectes americanus) was inflammation characterised by acute necrosis (Wolke, 1975). Chronically infected turbot (Scophthalmus maximus) exhibited characteristic skin lesions which progressed to ulceration and muscle necrosis (Richards, 1980); destruction of epidermis accompanied by haemorrhaging and necrosis of connective tissue was reported by Håstein (1974).

In turbot, the eye is particularly affected with oedema leading to detachment of the retina accompanied by infiltration of mononuclear leucocytes (Horne et al., 1977; Richards, 1980). Pronounced histopathological features of vibriosis are demonstrated in the major organs of the host with spleen, kidney and liver being severely affected. Splenic necrosis (Håstein, 1974; Harbell, 1976) with a concomitant destruction of haemopoetic tissues (Horne et al., 1977; Richards, 1980) and loss of melanomacrophage areas has been reported (Håstein, 1974; Horne et al., 1977). Histochemistry revealed the deposition of ferric (Fe^{+++}) iron in infected spleen tissue (Agius, 1979; Richards, 1980). The kidney appears necrotic (Harbell, 1976; Richards, 1980), renal tubules, glomeruli and haemopoetic tissues being particularly affected (Håstein, 1974; Richards, 1980), with

infiltration of macrophages (Hacking & Budd, 1971) and melanin containing cells (Håstein, 1974). As in the spleen, ferric iron deposition also occurs (Wolke, 1975; Richards, 1980). Infected liver tissue is characterised by necrosis of liver hepatocytes (Hacking & Budd, 1971; Håstein, 1974; Harbell, 1976; Richards, 1980) and general swelling of this organ.

1.2.5 Toxic products from V. anguillarum

Toxic products from V. anguillarum were first demonstrated by Umbreit and Ordal (1972) who found that culture supernatant contained a heat stable component which was toxic to goldfish (Carassius auratus). Umbreit and Tripp (1975) found heat-killed bacteria were toxic to goldfish and surprisingly, that culture supernatant from a 24 hr culture was more lethal than the same volume of culture. Harbell et al. (1979) found that cell-free supernatant had no toxic effects on coho salmon (O. kisutch).

Significant reductions in red blood cell number, haemoglobin and haematocrit have been reported in salmonids with vibriosis (Cardwell & Smith, 1972; Levin et al., 1972; Harbell et al., 1979). Wolke (1975) and Roberts (1976) implicated a haemolytic toxic substance as being responsible for the anaemia evident in infected fish. An important diagnostic feature of V. anguillarum is a zone of haemolysis observed around colonies cultured on blood agar. Considering that haemolytic products of this organism have been recognised since as early as 1927 (David, 1927), it is surprising that the haemolytic properties were not investigated to any extent until the work of Munn (1978). Munn found that the haemolytic component was produced during stationary phase of culture, had a pH optimum of 7.3 and its production was enhanced by the presence of bovine serum albumin (BSA). Munn (1980)

went on to partially purify the haemolytic components in culture supernatant and demonstrated that the kinetics of lysis involved a two stage process. A prelytic phase, believed to involve binding of haemolysin to the erythrocyte membrane, was followed by a phase during which actual cell lysis and release of haemoglobin occurred; the rates of both phases depended on the concentration of haemolysin. The binding site for the toxin was shown to be a ganglioside, however it was found not to be GM1, which is the binding site for V. cholerae toxin.

Unlike the heat-labile protein exotoxins, endotoxins are heat stable lipopolysaccharides associated with the outer membranes of Gram negative bacteria; their toxicity resides in the lipid moiety. Sub lethal doses injected into mammals cause fever, leukopenia, hyperglycaemia and haemorrhagic necrosis (Davis et al., 1980). The endotoxin of V. anguillarum was found to be non-toxic to salmonids (Abe, 1972; Harbell et al., 1979) with fish appearing to be generally tolerant of injected endotoxin (Berczi et al., 1966).

Examination of V. anguillarum for enzymes that may be potential aggressins has not been carried out in any detail. However, McCarthy et al. (1974) showed this organism to produce esterase, lipase, lecithinase and deoxyribonulease during in vitro culture.

1.2.6 Prevention of vibriosis

As with all infections of fish it is preferable to prevent vibriosis rather than attempt to cure it (Anderson & Conroy, 1970). Good husbandry is essential in disease prevention (Shepherd & Poupard, 1975), the use of disease-free eggs and fry, pelleted diets and clean, well-aerated water are important in maintaining disease-free conditions. In addition, a knowledge of environmental influences

known to affect fish detrimentally and consequently render them susceptible to disease should be carefully monitored and the necessary precautions taken.

Water temperature and oxygen tension appear to be important factors contributing to the onset of vibriosis (Ross, 1970; Fryer et al., 1972; Wood, 1974). Water temperature is directly related to oxygen tension such that a rise in temperature causes a concomitant lowering of the oxygen tension. Which of the parameters is of greater importance has not been clarified, however it would seem that a reduction in oxygen tension may lead to stress in the fish while an increase in temperature would shorten the generation time of the potential pathogen. In the event of a rise in ambient water temperature, stressing of fish by handling and over-crowding should be reduced to a minimum (Ross, 1970; Fryer et al., 1972; Wood, 1974), and feeding raw marine fish offal should cease. Kocylowski (1963) recommended, in the case of eels, that fish should be transferred to well aerated fresh-water and the stocking density reduced, under conditions of higher water temperature.

Although good husbandry is essential, culture systems employing for instance, floating net-pens (Novotny, 1978) can not be controlled to the same extent as purpose-built culture systems which may be protected from natural, possibly infected fish stocks. In addition, with an aim to improving fish production, suitable species are being cultured in heated waste water to increase growth rate (Aston & Brown, 1975; Aston et al., 1976; Ingram, personal communication). Under these conditions the growth of opportunist bacteria would be enhanced.

1.2.7 Protection against vibriosis by vaccination and the use of antibiotics

1.2.7.1 Vaccination

The literature available on vaccination as a means of preventing vibriosis discusses three main techniques of antigen administration: 1) oral, 2) by injection or 3) by immersion; and how the efficacy of a given vaccination protocol relates to antigen preparation.

Duff (1942) first considered oral immunisation in fish when a chloroform-killed preparation of Aeromonas salmonicida was fed to cut-throat salmon (Salmo clarkii), resulting in a consequent reduction in mortality when exposed to natural infections. Krantz et al. (1963) gave single injections of formalin-killed A. salmonicida to trout and demonstrated protection against a subsequent intraperitoneal challenge with viable bacteria.

Use of immunisation for the control of vibriosis was first attempted by Endo et al. (1961) by feeding a multivalent vaccine consisting of Vibrio piscium (V. anguillarum), A. salmonicida, Chondrococcus columnaris (Flexibacter columnaris) and Haemophilus piscium. A reduction of 60% in mortality was experienced during subsequent outbreaks of vibriosis. Harrel (1973) found that injecting rainbow trout (S. gairdneri) with either heat-killed or formalin-treated V. anguillarum produced agglutinating antibody in serum and mucus, heat-killed cells injected with complete adjuvant gave higher titres in serum (250,000). Baudin-Laurencin & Tangtrongpiros (1980) investigated the use of heat-killed cells, formalin-killed cells and bacterin composed of strains 775 and 1669 as prepared by Harrel et al. (1976). These preparations were administered to coho salmon by feeding, immersion and intraperitoneal injection, with the result that injection gave generally higher serum agglutination titres (mean = 142.6 for all three antigens) as compared to immersion (mean = 96 for all three antigens) and oral administration (mean = 41).

They further showed that the agglutination titre depended on the form of the antigen used such that heat-killed cells gave slightly higher titres when administered by immersion than by injection (90 and 82 respectively) although this was reversed when applying formalin-killed cells (115 and 186 respectively).

Antipa (1976) found significant reduction in mortality in chinook salmon (Oncorhyncus kishawytscha) following inoculation with either heat-killed (22.3% mortality), formalin-killed (37.8% mortality) or heat and formalin-treated (35.3% mortality) V. anguillarum when challenged by a natural epizootic by comparison with the observed mortality in control fish of 85%. In the same experiment, coho salmon (O. kisutch) were also vaccinated as above, but mortality in all groups, including control fish was low (c 5.5%) suggesting either natural resistance in coho salmon or species-specific strains of V. anguillarum. The work of Harrell (1973) was supported in that heat-killed bacteria gave better protection and higher agglutination titres (approximately 64). Harrell (1978) further demonstrated the efficacy of heat-killed cells in vaccination when he protected coho salmon against a natural outbreak of vibriosis by a single injection of 2-4 mg of preparation.

By injecting juvenile coho salmon with two strains of V. anguillarum, Schiewe et al. (1977) found that fish injected with one strain gave protection when challenged by a subcutaneous injection of viable bacteria of the other strain; however, better protection was afforded to fish receiving the homologous antigen. When vaccinated fish were exposed to a natural infection, the same result was experienced.

A comparison between oral and parenteral administration of heat-killed cells carried out by Rohovec (1974), showed chinook salmon

injected with the bacterin to have lower mortality (7%) as compared to orally administered vaccine (10%). Fryer et al. (1978) found a similar response but showed that oral immunization could be improved by feeding the antigen for longer and furthermore that ambient water temperature had little effect on oral vaccination. Nelson (1972) protected chinook salmon against vibriosis by oral administration of lyophilized, sonicated bacteria, reducing mortality by 70%. In addition he found that the amount of antigen administered was important; a reduction in vaccine dosage from 300 µg to 100 µg doubled the mortality but, elevating the dosage from 300 µg to 1000 µg did not reduce mortality. This feature would seem to be economically important when considering vaccination programmes; insufficient vaccine could give poor protection and could perhaps lead to tolerance in fish, whilst excess vaccine could constitute an unnecessary waste of money.

Croy and Amend (1977) compared intra-peritoneal injection with hyperosmotic infiltration to vaccinate sockeye salmon with a formalin-treated preparation. Several hyperosmotic regimes were followed, using 5.32% NaCl, 10 x Hanks balanced salt solution (HBSS) and 1 x HBSS with 8% NaCl as initial dipping solutions prior to dipping in bacterin solution. The results showed that injection gave generally better protection than hyperosmotic solutions, the 10 x HBSS gave better results.

Injection of vaccine as compared to hyperosmotic infiltration was also investigated by Antipa and Amend (1977). Intraperitoneal injection of formalin-killed bacteria was compared to hyperosmotic infiltration, carried out by placing fish in 20% urea containing bacterin, under vacuum. The results suggested that both techniques were effective although injected bacteria resulted in slightly lower mortality.

Amend et al. (1980) found direct immersion of steelhead trout (S. gairderi) into vaccine, incorporating a bivalent bacterin containing two strains of V. anguillarum, gave significant protection when exposed to natural infections. Because of the nature of the experiment they were unable to observe any homologous effects, as observed by Schiewe & Hodgins (1977).

The use of viable avirulent bacteria to vaccinate steelhead trout against virulent strains was investigated by Braaten and Hodgins (1976). Avirulent bacteria administered by oral and parenteral routes gave 98% and 100% protection respectively, when challenged with intraperitoneal injections of virulent bacteria.

Evelyn and Ketchson (1980), using a multivalent formalin-killed preparation of V. anguillarum, Vibrio spp. and A. salmonicida, examined the efficacy of administering combinations of various bacterins to sockeye, coho and chinook salmon. The results suggested that intraperitoneal administration was more effective in protecting these species and that the fish receiving homologous antigen were better protected. The efficacy of intraperitoneal injection was further supported by the work of Håstein et al. (1980) who reported that injection of formalin-killed bacteria gave better protection than injection or dipping using a commercial vaccine.

Research carried out concerning the potential of vaccination as a means of controlling fish disease has been rewarding, giving useful insight into ways of reducing mortalities incurred during fish culture. However this area of research appears to have been hampered by lack of conformity in the species, size and genetic stock of fish used. It was suggested by Egidius and Anderson (1975, 1978) and Antipa (1976) that species-specific strains of V. anguillarum occur. When vaccinated fish are challenged by 'natural' infections the extent

of the species specificity cannot be determined.

Fletcher and White (1973) found that in plaice, localised immunity occurred as a direct consequence of administration route such that oral antigens induced antibody production in the intestinal mucus while parenteral injection gave high serum titres. At present the means by which V. anguillarum enters the host is unclear, and a knowledge of invasion routes would assist in future vaccination programmes.

1.2.7.2 Antibiotics in prophylaxis and therapy

Antibiotics have been used for both prophylactic and therapeutic control of vibriosis, the main compounds being sulphonamides, nitrofurans and selected antibiotics. Although V. anguillarum has been shown to be sensitive to several sulpha-drugs (McCarthy et al., 1974; Jo et al., 1979), not all members of this group are suitable for the treatment of fish. For rapid uptake of drug from the gut or following injection, antibiotics have to be water soluble hence insoluble sulphonamides such as sulphaguanidine, phthalylsulphathiazole and succinylsulphathiazole are unsuitable. In aquaculture, the sulphonamide of choice would seem to be sulphamerizine, which is in widespread use in Japan (Hoshina et al., 1957; Saito et al., 1964) and North America (Ross, 1970; Bullock et al., 1971; Hodgins et al., 1977). The combination of two sulphonamides such as sulphadiazine and trimethoprim has been found by McCarthy et al. (1974) to be efficient against V. anguillarum, and is currently being used in commercial fish farms in the United Kingdom for this purpose.

Caution must be exercised in the use of sulpha-drugs as Johnson and Brice (1953) found that sulphamerizine was toxic to fingerling silver salmon, while Litchfield (1939) found sulphanilamide

to be more toxic to fish than frogs and chickens. Litchfield also found that growth of rainbow trout (S. gairdneri) was unaffected by continuous prophylaxis using sulphanilamide, whereas, in brook trout (Salvelinus fontinalis) growth rate was reduced in proportion to dosage.

Of the nitrofurans; nitrofurazone, nitrofurprinol, furazolidone and nifurprazine appear to be the main compounds used. Although predominantly used for prophylaxis (Hayashi et al., 1964; Roberts & Shepherd, 1974; Wood, 1974; Richards, 1980) they are also used for therapy (Kubota & Hagita, 1963). Literature concerning the toxic nature of nitrofurans and other antibiotics in fish therapy has not been encountered. In addition, the effects of antibiotics on fish, in relation to species, size and environmental conditions have been neglected areas of research. Litchfield (1939) found the toxicity of sulphanilamide in some fish to be temperature dependent, suggesting that environmental influences are important.

Strains of V. anguillarum have been shown to be sensitive to a number of antibiotics (Fryer et al., 1972; McCarthy et al., 1974; Jo et al., 1979) however, not all are suitable for aquaculture. The predominant antibiotics used are oxytetracycline and chloramphenicol (Ross, 1970; Bullock et al., 1971; Fryer et al., 1972; Roberts & Shepherd, 1974; Novotny, 1975; Sawyer & Strout, 1977; Novotny, 1978; Richards, 1980). Although normally administered by oral application as a means of prophylaxis, infected fish have to be injected with antibiotic, as diseased fish invariably cease feeding (Kusuda, 1966).

Sawyer and Strout (1977) compared vaccination with antibiotic medication for protecting coho salmon against vibriosis. Injection with killed cells gave better protection (2.9% mortality) as compared to feeding of oxytetracycline (6.7% mortality). In addition, they found

that fish receiving antibiotic or vaccine had significantly better growth rates than untreated controls. This was attributed to fish having to expend less energy in defence mechanisms and hence being able to direct more energy toward growth processes. The feeding of agricultural livestock with antibiotics in the diet causes increased weight gain in animals such as pigs and poultry (Jukes, 1973), and a similar situation may have been occurring in the above experiments. Jukes (1973) suggested that increased growth rate in agricultural animals was attributable to reduction of pathogenic gut bacteria. The possibility of fish having a true commensal flora has not been studied to any great extent; however, bacteria are invariably found in the intestines of fish.

Although the above antibiotics have been shown to be effective against V. anguillarum, some are active against both pathogenic and commensal bacteria; and consequently health and growth rates can be detrimentally affected by removal of gut flora from medicated fish (Kubota & Hagita, 1963). As shown by Sawyer and Strout (1977), medication can actually improve growth rates as well as protect fish from disease; it seems likely therefore that the effect of medication depends on the importance of a commensal gut flora to the host.

As a consequence of using antibiotics for the treatment of vibriosis and other fish diseases, strains of the causative organisms have developed resistance to some of the more widely used antibiotics. Kubota & Hagita (1963) reported that strains of a halophilic Vibrio spp had developed tolerance to chloramphenicol and sulphamethoxine; while Wood (1974) stated that sulphamerizine is no longer effective against V. anguillarum. In Japan, strains of this species carry resistance factors (R-factors) for sulphonamides, streptomycin, chloramphenicol

and tetracycline (Aoki et al., 1974) and are developing resistance to nitrofurans (Aoki et al., 1975).

Drug resistance has been reported in many of the major fish pathogens. Aeromonas salmonicida has been found to be resistant to terramycin and various sulpha-compounds (Novotny, 1978), sulphamerizine, (Post, 1965), sulphathiazole, tetracycline and chloramphenicol (Aoki et al., 1971). Resistance to sulphadimidine has been found in strains of A. liquefaciens (Neuman & Ploger, 1980). Examination of the gut flora of cultured carp revealed that a high percentage of strains of Aeromonas liquefaciens, Pseudomonas, Enterobacteriacae and Vibrio were carrying R-factors for sulphonamides and tetracycline (Aoki, 1974).

The drug resistance exhibited by these bacteria is of paramount importance, not only in aquaculture but also in the treatment of human diseases. Well established antibiotics are becoming ineffective, if not obsolete, for the treatment of major fish diseases. In addition, resistance has been developed to antibiotics commonly used in human medicine. Aoki et al. (1974) demonstrated resistance factors in naturally occurring strains of V. anguillarum to several antimicrobial agents, which were transferable in vitro to the human pathogen V. cholerae.

The resistance of major fish pathogens to antibiotics has been studied, but there is no information available on the resistance of bacteria which normally reside in marine and fresh water or in the digestive tracts of aquatic animals. These could carry R-factors to a range of antibiotics introduced into waterways by aquaculture, human sewage and/or farm effluents. A feature of particular concern lies in the fact that resistance of fish pathogenic bacteria to antibiotics was evident in the 1950's (Snieszko & Bullock, 1957) and yet antibiotics continued to be used ad libitum.

Examination of the published literature on vibriosis reveals certain areas of research to have been neglected. The paucity of detailed studies of vibriosis and the dearth of information available on the pathogen-host relationship prompted the present work to be undertaken. In summary, it is hoped that a rational approach to a better understanding of the interaction between this pathogen and its host will lead to improved methods of control.

CHAPTER 2. MATERIALS AND METHODS

2.1.0 CHEMICALS AND REAGENTS

Chemicals and reagents were obtained from BDH (Poole, Dorset) and Sigma Chemical Company (London), unless otherwise stated.

2.2.0 BACTERIOLOGY

2.2.1 Bacteria

Strains of Vibrio anguillarum were obtained from various geographical locations (Table 2) and maintained under liquid nitrogen storage using 10% $^{w}/_{v}$ skimmed milk (Difco) as protectant.

A strain of Klebsiella pneumoniae was obtained from the Department of Bacteriology, Greenbank Hospital, Plymouth; and a strain of Pseudomonas aeruginosa was obtained from the culture collection of the Department of Biological Sciences, Plymouth Polytechnic.

2.2.2 Media

Bacteria were routinely cultured on tryptone soy agar (TSA) with and without enrichment (5% sterile horse blood); and in tryptone soy broth (TSB) with agitation. Differential and selective media were used as outlined in Table 3. All media were adjusted to give a final NaCl concentration of 1.5g 100 cm^{-3}, and incubated at 25^{o}C.

2.2.3 Bacterial culture

From liquid nitrogen storage, strains were streaked on to TSA blood agar (2.2.2) and after checking for culture purity, subcultured to other media.

2.2.4 Bacterial identification

Salient bacteriological and biochemical tests used for the

Table 2 Source of strains

Strain	Geographical location	Initial host
3022A	Denmark	Unknown
UNH 569	USA	Unknown
HC 2	USA	Salmonid
PT-472	Japan	Unknown
PT-78069	Japan	Unknown
A1330/78	Norway	Trout
8.15	USA	Unknown
PT-78001	Japan	Ayu
NCMB 829	Scotland	Sea trout
MS 549	USA	Cod
775	USA	Salmon
HC7	England	Eel
NCMB 6	Denmark	Cod
NCMB 1873	USA	Chinook salmon
NCMB 1876	USA	Steelhead trout
A20/76	Norway	Trout
A555/76	Norway	Trout
COB 408	France	Trout
1669	USA	Salmonid
34/71	England	Eel
7/75	England	Eel
MS 2072	USA	Shrimp
PB-15	Japan	Unknown
PT-7601	Japan	Unknown
NCMB 407	Scotland	Plaice
NCMB 571	Japan	Rainbow trout
NCMB 572	Japan	Rainbow trout
NCMB 1291	Scotland	Saithe
NCMB 1336	USA	Clam
8.13	USA	Chinook salmon
8.14	USA	Coho salmon
15/75	England	Unknown
MS 424	USA	Unknown
MS 439	USA	Catfish
MS 463	USA	Unknown
MS 469	USA	Unknown
MS 974	USA	Chinook salmon
PO-103	Japan	Unknown

Table 3 Culture media and their use

Medium	Use	Reference
Differencial media		
Czapek Dox agar	Nitrate as sole nitrogen source	Oxoid Manual,1971
DNase agar	DNase production	Di Salvo,1958
Egg agar	Coagulase/lipase production	Lundbeck et al 1966
Elastin agar	Elastase production	Sbarra et al,1960
Eosin methylene blue agar	Lactose utilization	Levine,1921
Gelatin agar	Gelatinase production	Oxoid Manual,1971
Lipid agar	Lipase production	Hugo et al,1962
MacConkey agar	Growth in the presence of bile salts	Oxoid Manual,1971
Milk agar	Protease production	Brown et al,1970
Starch agar	Amylase production	Cowan & Steel,1974
Urea agar	Urease production	Oxoid Manual,1971
Selective media		
Bismuth sulphite agar	Growth in presence of $BiSO_3$	Oxoid Manual,1971
Lauryl agar	Growth in presence of SDS	Oxoid Manual,1971
Mannitol salt agar	Growth in presence of mannitol and NaCl	Oxoid Manual,1971
Potato dextrose agar	Potato extract and dextrose as sole nutrients	Oxoid Manual,1971
Staphylococcal 110 medium	Growth in presence of mannitol and high NaCl levels	Oxoid Manual,1971
TCBS Cholera medium	Selective for *Vibrio* spp	Oxoid Manual,1971
Tetrathionate agar	Growth in presence of iodine	Oxoid Manual,1971

identification of isolates were based on those of Hendrie et al. (1971).

2.2.5 Antibiotic sensitivity

Sensitivity of strains to antibiotics was determined by overlaying seeded agar plates (containing approximately 10^7 viable bacteria cm^{-3}) with antibiotics in the form of multodiscs (Oxoid). Following incubation (at 25°C, 24 hr), sensitivity was quantified by measuring the diameter of inhibition zones.

2.2.6 Bacterial enumeration

Viable bacteria in broth cultures were enumerated by serial dilution (10-fold) in peptone water (Oxoid, 1.5g 100 cm^{-3} NaCl) with subsequent culture on TSA using either the 'pour-plate' or 'drop-plate' (Miles et al., 1938) techniques. Following incubation (25°C), resultant colonies were counted using a colony counter (Don Whitley CC50). Viable bacteria in infected eel tissue were enumerated as follows:- Eels, pinned to a dissection board with flamed awls, were swabbed with tincture of iodine. Using flamed dissection implements, the body cavity was opened and salient tissues aseptically removed. After weighing in sterile weighboats, tissues were placed in a sterile mortar and ground up with peptone water (10 cm^3) and a little sharp sand. The resultant macerate was diluted and enumerated as above.

2.3.0 ANIMALS

2.3.1 Fish

Fish species, their source and general maintenance were as outlined in Table 4. For general laboratory experiments, fish were kept in perspex aquaria (37 x 26 x 21 cm) containing 4 dm^3 water, with aeration. Eels were prevented from escaping by means of perspex lids

Table 4 Source and maintenance of fish

Fish	Source	Maintenance
SCYLIORHINIDAE		
Lesser-spotted dogfish *Scyliorhinus caniculus*,L	Marine Biological Association of the United Kingdom(MBA)	Recirculating sea water at 10°C
ANGUILLIDAE		
European fresh-water eel *Anguilla anguilla*,L	Pudleigh Mill Fish Farm,Chard,Somerset; Slapton Ley,South Devon;The Lord Elliot Estate,St Germans,Cornwall.Hinkley Point Fish Farm.	Recirculating 50% sea water at 15°C
MERLUCCIDAE		
Hake *Merluccius merluccius*,L	MBA	Used at time of capture
GADIDAE		
Whiting *Merlangius merlangus*,L	MBA	Used at time of capture
Blue whiting *Micromesitius poutassou*,(Risso)	MBA	Used at time of capture
Ling *Molva molva*,L	MBA	Used at time of capture
Five-bearded rockling *Ciliata mustela*,L	Wembury beach,Devon	Recirculating 100% sea water at 15°C
BLENNIIDAE		
Blenny *Blennius pholis*,L	Wembury beach,Devon	Recirculating 100% sea water at 15°C
MUGILIDAE		
Thick-lipped grey mullet *Crenimugil labrosus*,(Risso)	Ford estuary, St Johns, Cornwall.	Recirculating 100% sea water at 15°C
BOTHIDAE		
Turbot *Scophthalmus maximus*,L	MBA	Used at time of capture
Brill *Scophthalmus rhombus*	MBA	Used at time of capture
PLEURONECTIDAE		
Dab *Limanda limanda*	MBA	Used at time of capture
Plaice *Pleuronectes platessa*,L	MBA	Used at time of capture
Flounder *Platichthys flesus*,L	MBA	Used at time of capture
CYPRINIDAE		
Carp *Cyprinus carpio*,L	Drakes Reservoir,Plymouth, Devon.	Recirculating fresh water

fastened by rubber bands. Fish were acclimated to required salinities for at least 5 days before use.

2.3.2 Amphibia

Specimens of the edible frog (Rana esculenta, L) were obtained from:- Xenopus Biological Supplies, Redhill, Surrey.

2.3.3 Mammals

Rabbits (New Zealand White) were obtained from:- Ranch Rabbits Ltd., Crawley Down, Sussex. Rats (Wistar) were bred at the Plymouth Polytechnic animal house. Guinea-pigs were obtained from:- Olac 76 Ltd., Bicester.

2.3.4 Molluscs

Specimens of the edible mussel (Mytilus edulis, L) were obtained from buoy chains in Plymouth Sound and maintained in aerated, recirculating sea water ($34^{o}/oo$).

2.4.0 INFECTION OF FISH

2.4.1 Experimental infection of fish

For most experiments fish were infected with vibriosis by intraperitoneal (ip) injection of approximately 10^{9} viable broth cultured bacteria, using a 25g hypodermic needle (Gilette) and a 1 cm^{3} syringe (Sterilin).

2.4.2 Fulfilment of Koch's postulates

Isolates of bacteria from infected eels, suggested by biochemical and bacteriological tests to be Vibrio anguillarum, were cultured in TSB prior to injection (ip) in to 'healthy' eels to fulfil Koch's postulates.

2.4.3 Confinement of disease

Vibriosis was confined to the laboratory by strict control measures. At the termination of experiments, infected fish were incinerated; and aquaria, nets and air stones disinfected in Hycolin (William Pearson Ltd., Hull).

2.5.0 HAEMATOLOGY

2.5.1 Blood sampling

2.5.1.1 Fish

Blood samples were taken from the dorsal artery of unanaesthetised fish, using a syringe (Sterilin) with a 25g hypodermic needle (Gilette). For the preparation of plasma, heparin (Sigma) was used as anticoagulant; while for sera, blood was allowed to clot at 4°C overnight and the sera separated by centrifugation (1000g, 15 min).

2.5.1.2 Mammals

Blood was taken from the marginal ear vein of rabbits by making an incision in the vein and collecting the blood in a sterile universal bottle. Rats and guinea-pigs were bled by cardiac puncture following anaesthesia with halothane (May & Baker).

Whole blood, plasma and sera were maintained at 4°C. For long term storage of plasma and sera, samples were kept at -20°C.

2.5.2 Haematocrit determination

To assess the effect of vibriosis on the blood of eels, haematocrit determinations were carried out based on the methods of Hesser (1960). Blood was drawn by capillary action into heparinised capillary tubes. After sealing one end with 'plasticine', tubes were centrifuged (2000g, 10 min) and the haematocrit recorded.

2.6.0 PROTEIN DETERMINATION

Protein determination was carried out using the method of Lowry et al. (1951) with bovine serum albumin (BSA) as standard protein. Samples for analysis were mixed with alkaline copper reagent, followed by the addition of Folin-Ciocalteau reagent; the resultant blue-grey colour was measured in a spectrophotometer (Cecil, @ 500 nm) against a set of BSA standards.

2.7.0 HISTOLOGY AND MICROSCOPY

2.7.1 Light microscopy

Tissues for wax histology were initially fixed in 10% $^{v}/_{v}$ formol PBS at 4°C. After approximately 2 hr tissues were cut into small pieces with a razor blade and placed into fresh fixative, thus enabling rapid and thorough fixation.

Whole eel preparations were fixed as above and cut into 4 mm slices. After decalcification in RDC (rapid decalcifier, Bethlehem Instruments Ltd), tissues were dehydrated in graded alcohols (70%, 5 hr; 90%, 2 hr; absolute, 2 hr x 2), cleared in xylene (2 x 1½ hr) and embedded in Fibrowax (Raymond Lamb). For routine work, 5 µm sections were cut using a rotary microtome; while for histochemistry 10 µm sections were used.

2.7.2 Histochemistry and routine staining

Wax sections were brought to water by treatment with xylene and graded alcohols and stained by the techniques outlined in Table 5. Semi-thin resin sections (approximately 1 µm) cut on a Porter-Blum ultramicrotome were placed onto glass microscope slides and stained with methylene blue.

Table 5 Histochemical Stains.

Staining procedure	Staining feature	Reference
Mallory's triple stain	General stain	Putt,1972
Giemsa stain*	General stain for blood cells.	Wrathmell[1]
Periodic Acid Schiffs (PAS)	Glycogen,mucoprotein mucopolysaccharide & glycolipid.	Pearse,1968
Alcian Blue (AB)	Sulphated muco-substances,acidic mucosubstances	Steedman,1950
Sudan Black (SB)	Lipid	Hayhoe & Flemens,1969
Perls' Prussian Blue	Ferric iron(Fe^{+++})	Drury & Wallington,1967

* Formula for Giemsa stain used:-

Giemsa buffer (BDH)	2 parts
methanol (BDH)	1 part
distilled water	8 parts

Dilute 3 volumes of above with 1 volume of Giemsa stain (BDH).

1. Wrathmell,personal communication.

2.7.3 Staining of bacterial flagella and capsules

Bacteria, mounted on acid-cleaned microscope slides were stained for the presence of flagella using the modified method of Cesares-Gill (Plimmer & Paine, 1921). Treatment with Kirkpatrick's fixative (ethanol, chloroform and formaldehyde) and after washing, treated with Plimmer's mordant (tannic acid, $AlCl_3$, $ZnCl_2$, basic fuchsin and ethanol) prior to staining with carbol fuchsin.

To determine the presence of capsules the method of Muir (Cowan & Steel, 1974) was employed. Heat-fixed smears were treated with carbol fuchsin and Muir's mordant ($HgCl_2$, potash alum and tannic acid), following washing the preparation was counter stained with methylene blue. Bacteria stain red, capsules stain blue.

2.7.4 Photography of slides for light microscopy

Slides were photographed with a Zeiss photo microscope using either 50 ASA (black and white) or 80 ASA (colour prints) 35mm film.

2.7.5 Electron microscopy

Considering the absence of data available on electron microscopical aspects of vibriosis and the causative organism, electron microscopy seemed a particularly pertinent technique to apply to a study of this disease.

2.7.6 Scanning electron microscopy

Materials for scanning electron microscopy (SEM) were fixed in 3% gluteraldehyde in PBS at $4^{\circ}C$; after approximately 2 hr samples were cut into smaller pieces and allowed to fix for at least 1 day in fresh fixative. Following post-fixing in 1% $^{v}/v$ osmium tetroxide in PBS (overnight), samples were dehydrated in graded alcohols and

critical point dried (Sam Dry PVT 3) using carbon dioxide as transitional fluid. Specimens were mounted on brass stubs by means of a quick-drying electroconductive paste (silverdag) and gold coated (approximately 14 nm) prior to observation in a Joel 35C Scanning Electron Microscope. Photographs were taken using FP4 film.

2.7.7 Transmission electron microscopy

Specimens for ultrathin sectioning were fixed in 3% $^{v}/_{v}$ gluteraldehyde in PBS at 4°C; after 2 hr, tissues were cut into smaller pieces and allowed to fix in fresh fixative for at least 1 day. Following post-fixation in 1% osmium tetroxide (2 hr) specimens were dehydrated in an alcohol gradient and treated with propylene oxide (EMscope) prior to gradual infiltration with resin (Spurr, 1974). Sections (approximately 100 nm thick) were cut using a Porter-Blum ultramicrotome with a glass knife and transferred to copper grids. These were stained with uranyl acetate (saturated solution, 15 min in total darkness) and after several washings in distilled water, stained with lead citrate (Reynolds, 1963) (15 min in the absence of CO_2).

Grids were observed using a Philips 300 transmission electron microscope at 80 kV and photographed using electron image plate film (Kodak).

2.7.8 Examination of *V. anguillarum* surface features by TEM

Bacteria examined for surface features were placed on carbon-stabilized, formvar-coated copper grids. Placing grids onto drops of culture caused bacteria to adhere to the formvar by static forces. Following brief rinsing in distilled water, specimens were either metal shadowed or negatively stained. Preparations were metal

shadowed with either gold or gold-palladium (40:60) at an angle of 20° or negatively stained with either 1% aqueous ammonium molybdate (pH 7.0) or 1% aqueous potassium phosphotungstate (pH 7.0) (Tweedy et al., 1968). Grids were observed using a Philips 300 TEM as outlined above (2.7.7).

2.7.9 Examination of bacteria for capsular material by TEM

The presence of capsular material around bacteria was observed by treating bacterial preparations with a reagent that causes capsular material to become electron dense and consequently visible by electron microscopy. Employing the method of Springer & Roth (1973) bacteria were fixed in fixative containing ruthenium red for 1 hr. This was followed by post-fixation in osmium tetroxide containing ruthenium red for a further 3 hr; after which time the material was processed for electron microscopy as described above (2.7.7). Both agar cultured and broth cultured bacteria were examined, with manipulation of broth cultured bacteria being facilitated by embedding in agar.

2.8.0 BIOCHEMICAL TECHNIQUES APPLIED TO THE STUDY OF V. ANGUILLARUM

2.8.1 Discontinuous polyacrylamide electrophoresis

Proteins were separated using the method of Laemmli (1970) in a vertical electrophoresis apparatus (Fig. 1). Polyacrylamide gels (16 x 14 cm) with an acrylamide : bisacrylamide ratio of 37.5:1 were employed. Proteins in sample buffer (10% glycerol, 5% mercaptoethanol, 3% SDS (sodium dodecyl sulphate), 0.01% bromophenol blue in 0.0625 M Tris pH 6.8) were separated in a Tris-glycine buffer system, containing 0.25% SDS (the detergent SDS dissociates proteins into their constituent polypeptide chains. Polyacrylamide gel electrophoresis

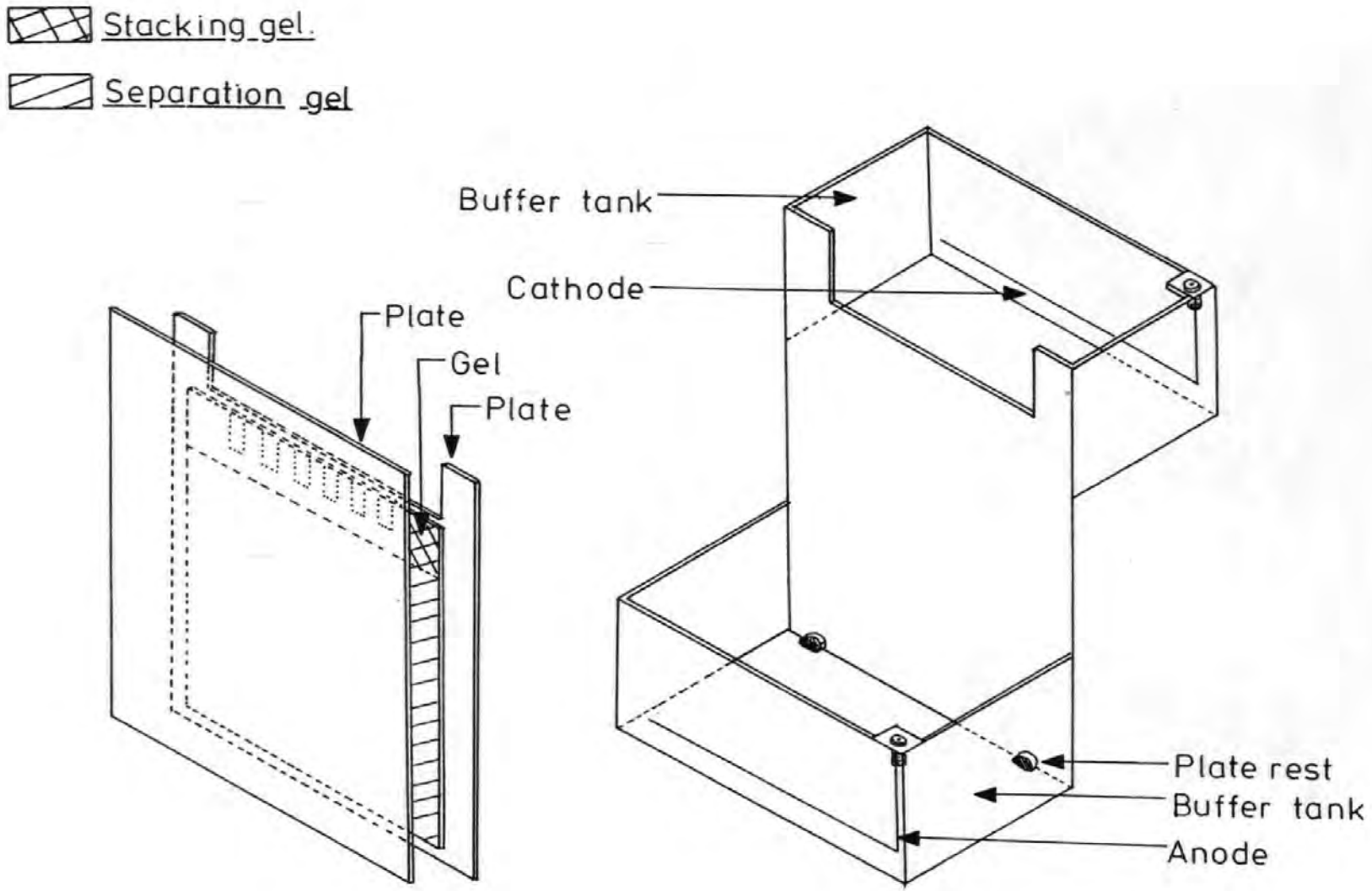


Fig 1 Vertical-slab electrophoresis apparatus

in the presence of SDS separates the polypeptide chains according to their molecular weight). By comparing test samples with standard protein preparations of known molecular weight (Pharmacia Ltd) the weight of unknown proteins could be determined. Proteins were applied to stacking gels (4.5% acrylamide) at 5-15 µg protein and 'stacked' for 1h at 10 mA, followed by separation (in 12.5% acrylamide) at 20 mA until the tracking dye was within 1 cm of the end of the gel. Gels were stained by the method of Weber & Osborn (1975) using Coomassie Brilliant Blue R (1.25g in 227 cm^3 methanol, 46 cm^3 glacial acetic acid made up to 500 cm^3 with distilled water) and excess stain removed by destaining (50 cm^3 methanol, 75 cm^3 glacial acetic acid made up to 1 dm^3). Following photography using a deep orange filter with background illumination (Churchwood & Holland, 1976) gels were dried on to absorbent white card at room temperature under vacuum. Molecular weights of proteins were extrapolated from a relative mobility versus log molecular weight graph of standard proteins.

2.9.0 METHODS FOR STUDYING THE PATHOGENESIS OF VIBRIOSIS

For the purpose of studying the pathogenesis of vibriosis in fish, eels were infected with broth cultured bacteria (2.4.1). At various time intervals during pathogenesis, eels were killed by decapitation and examined by histology (2.9.1) and bacteriology (2.9.2). The effect of vibriosis on the fish heart was determined by monitoring the electrocardiograph of eels during pathogenesis (2.9.3). The response of the eel host to bacterial infection was determined by examining the plasma iron levels before and after infection (2.9.4). Plasma proteins from infected and control eels were examined by electrophoresis (2.9.5).

2.9.1 Protocol for histopathology

Decapitated or moribund fish were placed on a dissection board with the ventral surface upwards and fastened by awls. Using forceps and scalpel, an incision was made along the length of the abdomen. Following the pinning back of the abdominal wall, salient tissues were removed and fixed for histology (2.7.0). Samples of gill tissues were obtained by severing the distal ends of the gill arch following the removal of the operculum. For the preparation of 'whole body' transverse sections, eels were cut into segments with a scalpel and fixed (2.7.1).

2.9.2 Bacteriological examination of fish

Fish to be examined for bacteriology were placed onto a surface pre-swabbed with tincture of iodine and fastened with flamed awls. Following swabbing of the ventral surface with tincture of iodine, the body cavity was opened with flamed dissection implements and tissues removed for qualitative bacterial assessment (2.2.2) and/or quantitative assessments (2.2.6).

2.9.3 Electrocardiography of vibriosis in eels

To elucidate the effect of vibriosis on the fish heart, eels were connected to an electrocardiography apparatus, and monitored before and after routine infection (2.4.1).

Electrocardiography was carried out using the technique of Roberts et al. (1973). Eels, anaesthetised by immersion in water containing 0.025% t-chlorbutol (Williams Ltd., London) were placed ventral surface upwards. Anaesthesia was maintained by wrapping the eels in tissue paper soaked in 0.025% chlorbutol. Electrodes were pushed through the skin on either side of the heart and positioned

whilst observing the electrocardiogram, once in position the electrodes were glued in place with quick setting adhesive (Eastman Ltd). The ECG was transmitted by a Dences SNR102F transmitter to a Dences 112R receiver connected to an oscilloscope (Solartron) and pen-recorder (Washington). The transmitter was placed inside a polythene bag on top of a polystyrene raft, to keep the system dry. Outside radio-interference was excluded by casing the apparatus in an earthed foil screen. The apparatus was as shown in Fig. 2.

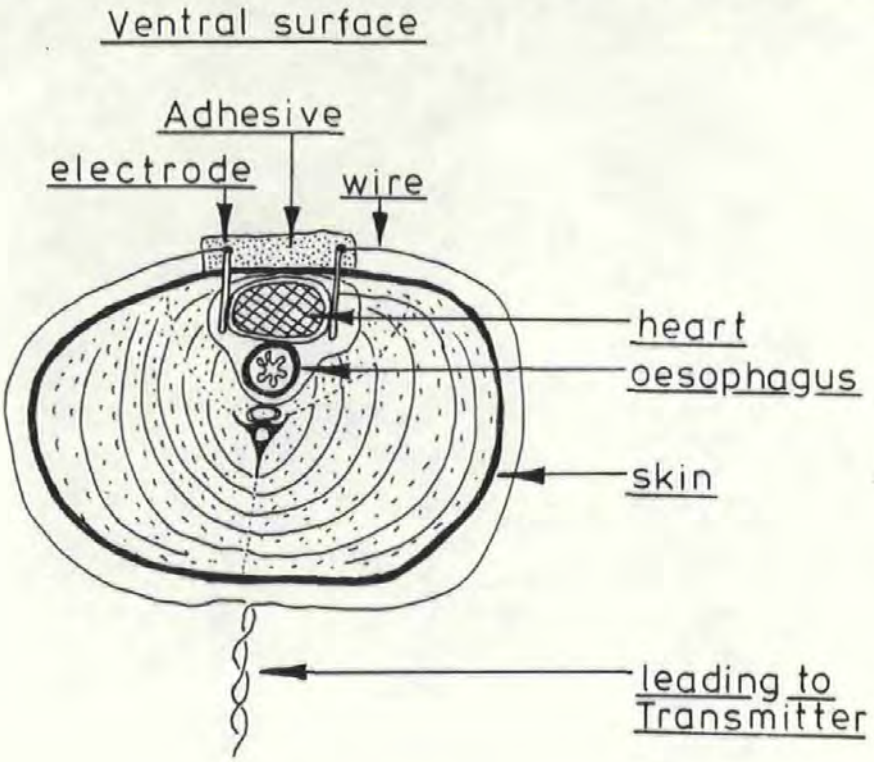
2.9.4 Determination of serum unsaturated iron binding capacity (UIBC)

The response of eels to infection with V. anguillarum, with respect to iron status was investigated by measuring the UIBC of eel sera/plasma using a Sigma Diagnostic Kit No. 565. At a slightly alkaline pH, ferrous ions, when added to a serum specimen, bind specifically to transferrin at the unsaturated iron-binding sites. Remaining unbound ferrous ions are reacted with ferrozine to form a magenta coloured complex with an absorption maximum at about 560 nm. The difference between the amount added is equivalent to the quantity bound by transferrin, termed the 'unsaturated iron-binding capacity'.

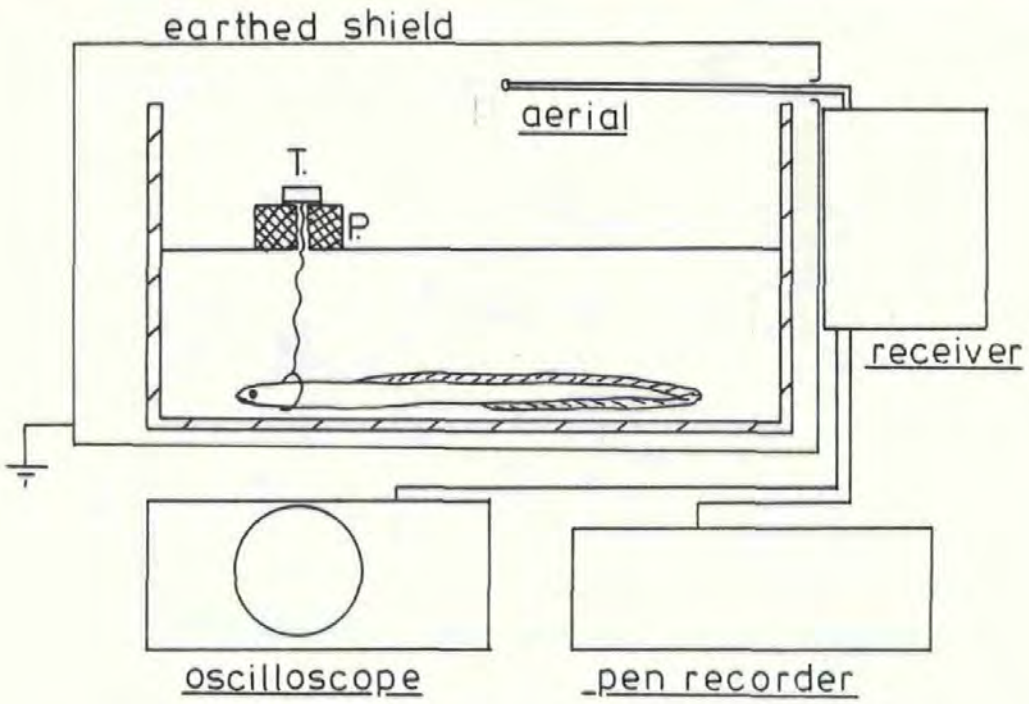
2.9.5 Electrophoretic analysis of plasma from infected and control eels

To elucidate the effect of infection on plasma proteins, samples of plasma from treated and control fish were electrophoretically separated in the presence and absence of SDS. Plasma samples were mixed with electrophoresis loading buffer (1 part plasma: 7 parts loading buffer); 10 µl aliquots were loaded into sample wells and the constituent proteins separated by electrophoresis (2.8.1). Proteins separated in the presence of SDS were compared with concurrently run

Fig 2 Apparatus for the monitoring of the electrocardiogram in eels.



a) Diagrammatic representation of eel showing location of electrodes.



b) Experimental apparatus (T=transmitter, P=polystyrene raft).

standard proteins of known molecular weight, while proteins separated in the absence of SDS were compared to a sample of human plasma.

2.9.6 Effects of the immunosuppressant cyclophosphamide on the inflammatory response in eels

In connection with studies concerning gut epithelial detachment during pathogenesis of vibriosis, attempts were made to suppress the inflammatory response in eels. The commercial preparation 'Endoxana' (W & D Pharmaceuticals) was injected (ip) at a rate of 15 mg Kg^{-1} body weight every 2 days for 2 weeks and with an inoculum of broth cultured *V. anguillarum* (UNH 569) containing 10^8 viable bacteria cm^{-3}. Control eels received sterile TSB instead of bacteria. Dead fish were fixed for light microscopy (2.7.1) and examined for bacteriology (2.2.4), while control fish were killed and fixed after 1 week.

2.9.7 Location of phagocytic areas in the eel

In an attempt to reveal the phagocytic areas of the eel, fish were injected (iv) with colloidal carbon (5mg) suspended in PBS. After 2 days, fish were killed by decapitation and fixed for histology (2.9.1). Sections were stained routinely (2.7.1) and examined by light microscopy.

2.9.8 Ionic analysis of eel plasma and other body fluids

A feature of vibriosis in fish was the presence of fluid in the gut. The nature of this fluid was examined by analysing for presence of key electrolytes, and comparing the data with the normal values obtained from uninfected eel plasma. Sodium and potassium concentrations were determined using a ILL Flamephotometer 343, while chloride was analysed using a Corning Chloride Meter 920.

2.10.0 METHODS FOR STUDYING ENTRY INTO THE FISH HOST BY V. ANGUILLARUM

With an aim to elucidating the route(s) by which V. anguillarum enters the fish host, fish were challenged with V. anguillarum by immersion in contaminated water (2.10.1), by injection of bacteria via various routes (2.10.3) and by feeding contaminated diet (2.10.2). The possibility of V. anguillarum being a gut commensal was examined by monitoring the entry of water-borne bacteria into the gut during osmoregulatory processes (2.10.4), by observing the response of this organism to conditions experienced in the gut (2.12.8) and by determining the ability of V. anguillarum to attach to the gut (2.12.7). In addition, the fate of bacteria inoculated into the gut was followed (2.10.5). Commensal aspects of V. anguillarum were related to bacteriological studies of local coastal fish (2.10.6).

The ability of V. anguillarum to traverse eel skin was also assessed (2.10.7). Bacterial isolates were identified as described above (2.2.4).

2.10.1 External challenge with V. anguillarum

In order to determine whether V. anguillarum could infect fish immersed in water containing viable bacteria, eels (50-60 gm wt) acclimated to 50% sea water were challenged by V. anguillarum in the surrounding water (10^6 viable bacteria cm^{-3}). Fish were maintained in contaminated water for one week prior to transfer to clean water for a further three weeks. Control fish were exposed to water inoculated with sterile TSB. The above experiments were repeated and fish were killed after 2, 7 and 14 days. Tissues, removed aseptically, were quantitatively assessed for bacteria (2.2.6) and fixed for histology (2.7.0).

Grey mullet (1.0 - 1.5 g wt) were subjected to the initial

challenge protocol as above but exposure duration was varied. Fish were placed into contaminated water for 30 min, 1 hr, 2 hr or 3 hr prior to maintenance in clean water for observation. Dying fish were fixed for histology and examined by bacteriology.

2.10.2 Feeding fish with contaminated diet

Blennies (mean wt. = 53.4 g) and rockling (mean wt. = 42.1 g) were conditioned to consume small, whole grey mullet. After one month, these fish were fed freshly killed mullet, injected with approximately 10^8 viable broth cultured bacteria. Feeding with contaminated fish continued for a total of five weeks after which normal feeding was resumed.

A second series of experiments involved the feeding of infected mussels to grey mullet. Mussels were infected by inoculating holding tank water with broth cultured *V. anguillarum* (c 10^6 cm^{-3}, UNH 569), which when accumulated led to their eventual death. Dead mussels were removed from their mantles, washed in fresh sea water and stored at -20°C. Bacterial examination of mussel tissue revealed the presence of *V. anguillarum*. Grey mullet maintained in 50% sea water, were fed the contaminated tissue for a period of one week followed by a two week period fed uncontaminated food. Control fish received uninfected mussels.

2.10.3 Effect of inoculation route on vibriosis in eels

To determine the effect of inoculation route on vibriosis in eels, fish (50-60 g wt) were injected with approximately 10^8 viable bacteria (UNH 569) by intramuscular (im), intraperitoneal (ip), intravascular (iv) and subcutaneous (sc) routes. In addition, inocula were introduced into the foregut and hindgut by means of a

catheter. Fish were also challenged with viable bacteria in the surrounding medium after receiving a 1 cm lesion (with a sterile scalpel) in their flank. Control fish received sterile TSB.

2.10.4 Entry of *V. anguillarum* as a consequence of osmoregulation

The extent to which suspended material in the surrounding medium entered fish was determined by acclimating eels and mullet to a range of salinities (2.3.1). A black particulate material (Aquadag, BDH), added to the water, enabled suspended matter entering the fish to be monitored by dissection of fish intestines. In concurrent experiments, eels were challenged with viable bacteria (UNH 569) in the holding tank water (10^6 cm^{-3}).

2.10.5 Fate of intestinal *V. anguillarum*

The fate of intestinal bacteria was examined by inoculating the hindgut of nine eels with approximately 10^6 viable bacteria (UNH 569) by means of a catheter. At 2, 7 and 14 days post-inoculation, three fish were killed by decapitation and their intestines fixed for electron microscopy (2.7.7). The fixed guts were cut into 5 mm segments which were further halved longitudinally, such that one half could be examined by SEM and the other by TEM (2.7.5).

2.10.6 The status of *V. anguillarum* in wild fish

Existing data concerning the microbiology of the aquatic environment has revealed that water-borne microbes are an integral part of the aquatic food chain. Members of the *Vibrionaceae*, including *V. anguillarum* have been shown to reside in various levels of this food chain, which includes fish (see literature review). For assessment of the status of *V. anguillarum* in coastal fish, specimens

of hake, blue whiting, whiting, ling, brill, dab, plaice and flounder, were caught by the MBA research vessel 'Squilla' in local coastal waters. Shortly after capture, fish were swabbed with tincture of iodine and samples of gut, kidney, spleen, liver and bile aseptically transferred to blood agar plates. Haemolytic colonies were subcultured and examined for motility, Gram reaction and sensitivity to O/129.

2.10.7 Penetration of eel skin by water-borne bacteria

The ability of bacteria to penetrate eel skin was determined by using freshly excised eel skin as a water-tight barrier between two chambers containing sterile 50% sea water on the inner surface of the skin, and water containing viable bacteria (10^6 cm^{-3}), on the outer surface (Fig. 3).

Qualitative assessment of penetration was determined by streaking water from the 'sterile' chamber onto TSA, following exposure to the skin for 12 hr and examining the skin by electron microscopy. Initial experiments to determine the presence of leaks in the apparatus were carried out by having unequal liquid levels in the chambers and observing the change in levels. During the experimentation proper, the liquid levels were equal; with the reaction of *V. anguillarum* compared to *Pseudomonas aeruginosa*. The extent to which the skin degenerated was examined by electron microscopy and compared with freshly fixed preparations.

2.11.0 METHODS FOR STUDYING THE FACTORS INFLUENCING ONSET OF VIBRIOSIS

To elucidate how environmental variables influence the onset of vibriosis, eels were infected (2.4.1) and exposed to changes in ambient temperature (2.11.1), salinity (2.11.2) and other possible stressing factors (2.11.3). In addition, the effect of fish weight

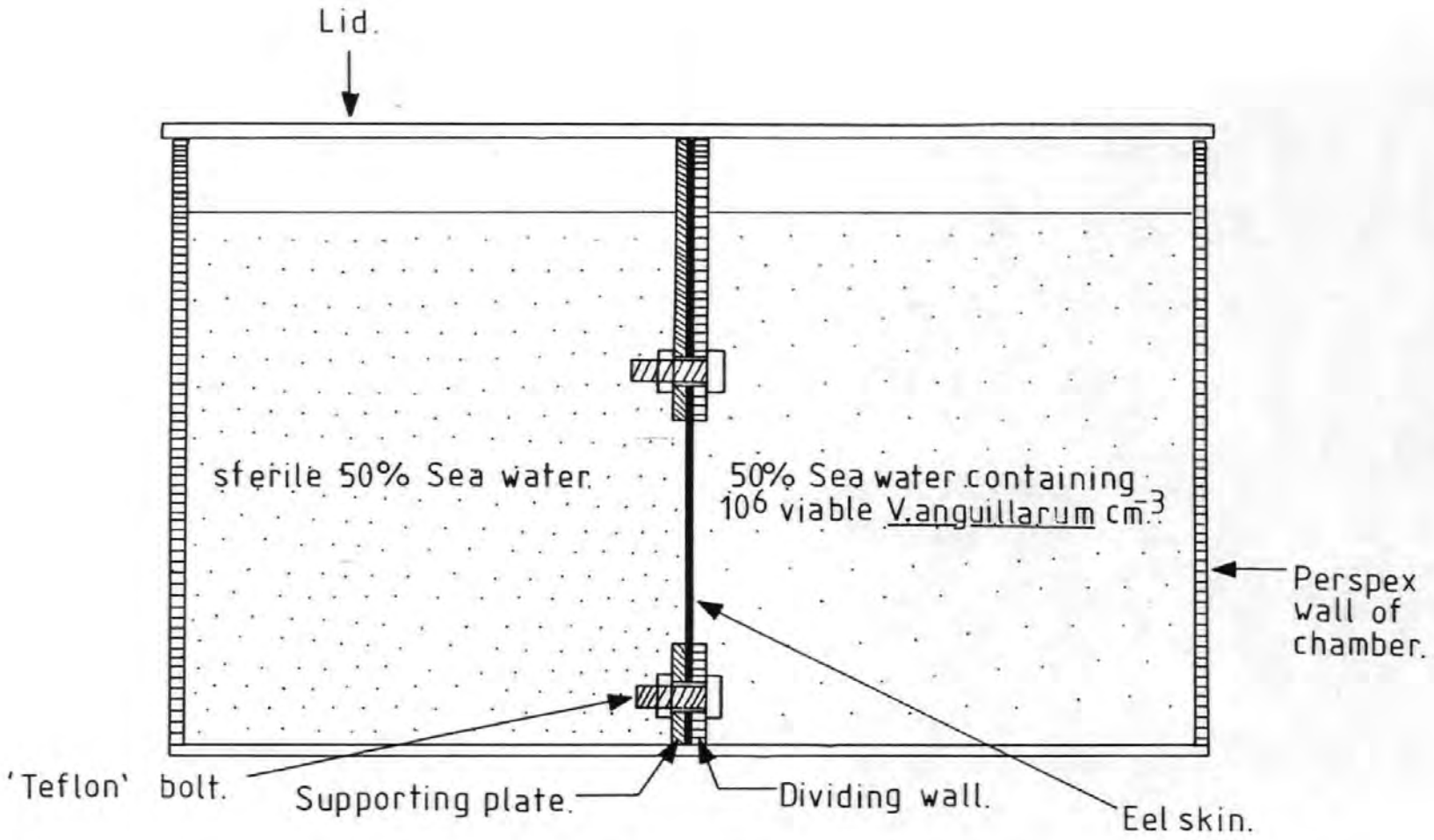


Fig 3 Apparatus used for the qualitative assessment of eel skin traversal by V.anguillarum

(2.11.4) and inoculum size (2.11.5) on pathogenesis was examined.

2.11.1 Effect of environmental temperature on pathogenesis

To determine the effect of environmental temperature on the pathogenesis of vibriosis, groups of nine eels (50-60 g wt) were acclimated to a range of ambient temperatures consisting of 5, 10, 20,25 and 37^{o}C. Following acclimation (7 days) six fish from each group were infected (2.4.1) while remaining fish received TSB. Mortalities were recorded daily; after 30 days the experiment was terminated and the mortality data processed.

2.11.2 Effect of water salinity on vibriosis

To elucidate the effect of environmental salinity on the pathogenesis of vibriosis, six eels from a stock of fish (mean wt. = 36.7 $\pm$ 7.6 g) were acclimated to a range of salinities (2.3.1) consisting of 0,17.0 $^{o}/_{oo}$ or 35.0 $^{o}/_{oo}$. Three fish from each salinity were infected (2.4.1) while control fish received TSB. After one week, the experiment was terminated and the mortality data processed.

2.11.3 Effect of other environmental influences on pathogenesis

Eels were infected with vibriosis (2.4.1) prior to being maintained in unaerated water or in 50% sea water deliberately polluted by the addition of powdered fish food to make the water cloudy. In addition, eels acclimated to freshwater were plunged into full-strength sea water. Observations were noted throughout the experiments and the mortality data processed at termination of the experiment (1 week).

2.11.4 Relationship between fish weight and susceptibility to vibriosis

The relationship between fish weight and susceptibility to vibriosis was determined by taking a group of 14 eels (mean wt. = 27.4 ± 20.7 g) and inoculating them with a broth culture (10^9 cm^{-3}) at a standard volume per unit weight (2 cm^3 Kg^{-1} body weight). Fish were maintained in 50% sea water and death time and cause of mortality (2.2.2) determined.

2.12.0 METHODS USED FOR THE ELUCIDATION OF POSSIBLE PATHOGENIC MECHANISMS AND VIRULENCE DETERMINANTS OF V.ANGUILLARUM

2.12.1 Determination of lethal dose -50 (LD-50)

The lethal dose 50 values of selected strains were determined by serially diluting stock bacterial cultures in peptone water (10-fold) and subsequently injecting batches of three fish with 0.1 cm^3 of each dilution. Viable counts were carried out on the stock broth cultures and the respective dilutions (2.2.6); mortalities were checked twice daily for one week post-inoculation, and the lethal dose 50 calculated using the method of Reed & Meunch (1938). The causative organism was isolated in each case and identified (2.2.4).

2.12.2 Enhancement and attenuation of virulence

For the purpose of elucidating intra-strain variation in

pathogenicity some strains were attenuated by repeated subculture on TSA (x 20), or had their virulence enhanced by passage through eels (6 hosts).

2.12.3 Fate of virulent and low-virulence strains in eels

To monitor the behaviour of V. anguillarum in vivo following infection (2.4.1), strains of varying virulence (2.12.1) were injected into groups of eels (50-60 g wt). Fish were killed by decapitation at time intervals, and bacteria in salient tissues quantified (2.2.6).

2.12.4 Preparation of eel phagocytes for in vitro phagocytosis studies

A feature of some bacterial pathogens is the ability to avoid being phagocytosed by host phagocytes (Smith, 1977). To elucidate the V. anguillarum-host phagocyte relationship, eel splenic macrophages were incubated with fluoresceinated V. anguillarum (2.12.5) and observed by ultra-violet microscopy. Splenic macrophages were obtained by teasing apart freshly dissected eel spleens in a tissue culture plate containing Eagle's medium (Difco). Following adherence (1 hr), splenic parenchymal cells and non-phagocytic blood cells were washed away with fresh medium. Observation of phagocytic cells was facilitated by fixing the cells in methanol (5 min) and staining with Giemsa (5 min).

2.12.5 Fluorescent staining of V. anguillarum

For use with in vitro phagocytosis studies (2.12.4), V. anguillarum was stained with a fluorescent compound using the method of Gelfand et al. (1976). Broth-cultured bacteria were heat-killed (60°C, 30 min) and washed three times in PBS. To one volume of washed suspension was added 5 volumes of 0.5 M carbonate/bicarbonate buffer and 2 volumes of

0.03% fluorescein isothiocyanate isomer 1 in the same buffer. Following incubation (2 hr, room temperature) the bacteria were washed three times in veronal buffer with 0.15 mM Ca, 2 mM Mg and 0.1% gelatin, and suspended in the same mixture. Aliquots of the fluoresceinated bacteria were stored at -20°C.

2.12.6 Response of V. anguillarum to non-specific agglutinating antibody in eel plasma

The presence of naturally occurring, non-specific agglutinating antibodies has been demonstrated in fish; these antibodies play a role in agglutinating antigenic matter including bacteria (Ellis, 1978). To examine the effect of eel agglutinins on V. anguillarum strains, volumes of serially diluted (doubling) bacterial broth culture were added to an equal volume of freshly prepared eel plasma in a microtitre plate. After rocking the plate by hand (10 min) the presence of agglutination was examined by light microscopy.

2.12.7 Ability of V. anguillarum to agglutinate erythrocytes

A prerequisite for infection resides in the ability of the invading organism to attach to mucous membranes (Smith, 1977). A technique that has been widely used to reveal the ability of bacteria to attach to eukaryotic cells is that of haemagglutination (Duguid & Gillies, 1958). The ability of various sugars to inhibit haemagglutination has also been used to elucidate the nature and specificity of the receptors on the eukaryotic cell surface (Old, 1972). The methodology of Trust et al. (1980) was used to examine the ability of V. anguillarum to agglutinate a range of erythrocytes.

Broth-cultured bacteria were washed, and resuspended, in Dulbecco phosphate-buffered saline. Erythrocytes, were washed using isotonic PBS

and resuspended to a 3% $^{v}/v$ suspension. To 20 µl of bacterial suspension, was added an equal volume of blood cell preparation on a cavity slide, after 10 min of gently rocking the slide, agglutination was assessed by microscopy. Sugar inhibition was tested for by performing the agglutination procedure in the presence of 20 µl of sugar solution (1% $^{w}/v$).

2.12.8 Effect of pH and fish bile on V.anguillarum and its relationship with virulence

In order to examine the response of V.anguillarum to conditions experienced in the fish gut, strains were cultured in a range of hydrogen ion concentration and in the presence of bile. For the response to pH, strains were cultured in TSB adjusted to a range of pH, of 4.0 - 10.0. After incubation (25^{o}C, 20hr) growth was assessed by measuring the absorbance (@ 590 nm) . The tolerance to bile was assessed by placing fresh fish bile into wells (No 1 cork borer) cut in seeded agar plates, and incubated (25^{o}C, 20 hr).

2.12.9 Effects of V.anguillarum cell wall material

To elucidate the possible effect of cell wall material from strains of varying virulence, eels (50-60 g wt) were injected (ip) with the sonicated preparation from approximately 10^{8} viable bacteria. Broth cultured bacteria were sedimented (35 000g, 30 min, 4^{o}C) and washed in 10 mM Tris-HCl pH 8.0. Washed pellets were resuspended in PBS (1.05% NaCl) and sonicated for 3 min (MSE: series PR-189) in 30 sec bursts with intermittent cooling. Preparations were checked for sterility by culture on TSA following passage through a Millipore filter (0.4 µm).

2.12.10 Effects of extra-cellular products of V.anguillarum

To examine the effect of culture supernatant on eels, cell-free supernatants were prepared (35 000g, 30 min, 4°C, membrane filtration). Eels (50-60g wt) were injected (ip) with 0.1 cm^3 of the preparation and examined after 1 day and 1 week for general necropsy.

To examine the effect of a crude haemolysin preparation on eels when injected (iv) with this preparation, fish were given low (300 haemolytic units, HU) and high (1000 HU) doses of haemolysin preparation. Tissue samples were fixed for histology (2.7.0) and blood samples taken for haematology (2.5.0).

A partially purified preparation of V.anguillarum haemolysin precipitated from broth culture (peptone,yeast extract,K_2HPO_4 medium) by ammonium sulphate, and antisera raised in rabbits to the above were kindly donated by Dr C.B.Munn.

2.12.11. Haemolysin production by V.anguillarum

To examine the qualitative production of haemolysin strains of varying virulence, salient strains were cultured on blood agar (2.2.2). Quantitative haemolysin production was assessed by relating colony diameters with the diameters of haemolysis zones, obtaining a zone diameter : colony ratio.

2.12.12 Analysis of plasma from infected eels for haemolysin using immuno-electrophoresis

The importance of immuno-electrophoresis for the detection of very small amounts of antigenic material has been well recognised (Owen,1980). To ascertain whether haemolysin(s) were produced in fish during the pathogenesis of vibriosis, infected eel plasma was

electrophoresed into agarose gels containing antisera produced in rabbits against a crude haemolysin preparation. Crossed immuno-electrophoresis was carried out as described below (2.12.28), while rocket immuno-electrophoresis was carried out based on the methods of Weeke (1975). Rabbit anti-haemolysin antiserum was added to 50 cm^3 of 1% ($^w/v$) agarose in sodium barbital buffer (at 55°C), and poured onto a glass plate (26 x 12.5 cm) and allowed to set. Plasma samples, doubly diluted in the above buffer, were applied onto the gel by placing 10 µl of each dilution into wells cut in the agarose matrix (3 mm diameter). The plate was placed into an electrophoresis apparatus (LKB Multiphor) and run for 2.5 hr at 8-10 volts cm^{-1}. Following electrophoresis, gels were washed in PBS and compressed between sheets of filter paper (15 min) and stained with Coomassie Blue R and destained (2.8.1). Plasma samples were run with diluted haemolysin preparation, as standards.

2.12.13 Enzyme production by V.anguillarum

To examine the enzymes produced by V.anguillarum and examine their possible relationship to pathogenesis and virulence, strains were cultured on specific substrates and the extent of utilization assessed. Initial qualitative examinations were carried out by streaking cultures on to media containing substrate (Table 3) and incubating (20 hr, 25°C). Quantitative estimates were obtained by placing cell-free broth culture supernatants (35 000g, 30 min, 4°C, membrane filtration) into wells cut into media (0.1 cm^3) and measuring zones of substrate utilization after incubation (20 hr, 25°C).

To examine the production and properties of proteases, bacteria were cultured in TSB and protease activity determined using casein as substrate, based on the method of Shieh & MacLean (1975). Cell-free

supernatant was allowed to react with the substrate (10 min) followed by precipitation of remaining protein with TCA (5%) and the resultant proteolysis determined using the method of Lowry et al (1951).

2.12.14 Elucidation of enzyme production in vivo by histochemistry

To elucidate enzyme production by V. anguillarum during pathogenesis in eels, infected and control eel tissues were fixed for histology (2.7.1) and examined by histochemistry (2.7.2).

2.12.15 Examination of colonial morphology with respect to virulence, in V. anguillarum

To ascertain if V. anguillarum colonial morphology may have a link with virulence, strains were cultured on TSA with and without enrichment (2.2.2), and the resultant colonies examined by transmitted and incident illumination.

2.12.16 The ultrastructure of V. anguillarum

At the time of writing this dissertation, the ultrastructure of V. anguillarum had not been examined. To examine the ultrastructure of strains of varying virulence, the above methodology was followed (2.7.5 - 2.7.8).

2.12.17 Preparation of V. anguillarum cell envelope Sphaeroplasts

To enable the envelope proteins to be examined by electrophoresis, the cell envelope was separated from the cytoplasm. Cell wall rigidity was reduced by removal of cellular peptidoglycan by treatment with lysozyme causing sphaeroplasts to be produced. These were disrupted by sonication allowing the bacterial envelope to be separated. For sphaeroplast production, the method of Smyth et al. (1976) modified by

Owen (personal communication) was adapted for use with V. anguillarum as follows:-

Cells were sedimented by centrifugation (38,000g, 30 min, 4°C) and resuspended in 10 mM Tris-HCl pH 8.0. Following sedimentation, washed cells were resuspended in 20% w/v sucrose in 30 mM Tris-HCl pH 8.0. Lysozyme in 30 mM Tris-HCl pH 8.0 containing 220 mM EDTA (disodium salt) was added to give a final concentration of 70 μg cm^{-3}. The mixture was kept at 0°C for 45 min for sphaeroplast formation to take place at which time DNase (from bovine pancreas, Sigma) was added to give a final concentration of 100 μg cm^{-3}. Sphaeroplast formation was observed by phase contrast microscopy of a wet mount preparation. Resultant sphaeroplasts were sedimented by centrifugation (10,000g, 30 min, 4°C) and resuspended in 20% w/v sucrose in 3 mM EDTA pH 7.2 containing DNase to a final concentration of 100 μg cm^{-3}. This suspension was subjected to sonication (MSE : series PR-189) given at 4 x 30 sec bursts of maximum energy with intermittent cooling. The extent of cell disruption was monitored by microscopy. An equal volume of 3 mM EDTA pH 7.2 was added and any remaining whole cells sedimented by centrifugation (2,000g, 15 min, 4°C). The supernatant containing ruptured sphaeroplasts and cytoplasmic debris was subjected to ultracentrifugation (48,000g, 1 hr, 4°C) to sediment envelope sphaeroplasts. The pellet was washed once in 10% w/v sucrose in 3 mM EDTA pH 7.2 and stored at -80°C.

2.12.18 Separation of outer and cytoplasmic membranes

Separation of outer and cytoplasmic membranes was carried out on cell envelope preparations (see above) using the detergent Sarkosyl (Ciba-Geigy), based on the technique of Filip et al. (1973). The resultant envelope preparation from 500 cm^3 TSB culture was incubated

with 1 cm^3 of 0.05% Sarkosyl ($^v/v$) for 1 h at room temperature, with separation of the insoluble outer membrane carried out by centrifugation (170,000g, 3 h, 4°C).

2.12.19 Extraction of V. anguillarum outer membrane proteins using lithium acetate

For the purpose of electrophoretic separation and examination of V. anguillarum outer-membrane proteins, broth cultured bacteria were treated with lithium acetate to extract the outer membrane proteins using the method of Heckels (1977).

Broth (TSB) cultured bacteria were sedimented by centrifugation (35,000g, 30 min, 4°C) and washed twice in PBS. A bacterial mass was suspended in 0.3M lithium acetate to give a concentration of about 2% $^w/v$. The mixture was stirred for 2.5 hr at 45°C and homogenised for 1 min in vortex mixer. Any whole cells were removed by centrifugation (2,000g, 15 min, 4°C) and the remaining supernatant centrifuged (15,000g, 20 min, 4°C) to remove cell debris. The final supernatant was dialysed against distilled water (2 days, 4°C) and the dialysed suspension freeze dried (Edwards Modulo) and stored in a desiccator. Electrophoresis was carried out as described above (2.8.1).

2.12.20 Extraction of outer membrane proteins using Triton X-100

Broth cultured bacteria were treated with Triton X-100 to extract the outer membrane proteins using the method of Archer (1979). Broth cultured V. anguillarum were sedimented by centrifugation (35,000g, 30 min, 4°C) and washed twice in PBS. The washed pellet was resuspended in 1% Triton X-100 in PBS (100cm^3 gm^{-1} bacteria) and incubated at 20°C (1 hr). After incubation, intact bacteria were removed by centrifugation (35,000g, 10 min, 4°C) and the supernatant dialysed

against distilled water (2 days, 4°C). Protein was concentrated by freeze drying and stored in a desiccator. Electrophoresis was carried out as outlined above (2.8.1).

2.12.21 Extraction of outer membrane proteins using guanidine hydrochloride

Broth cultured bacteria were extracted using guanidine hydrochloride, employing the method of Kabir (1977). Broth cultured bacteria were sedimented by centrifugation (35,000g, 30 min, 4°C) and washed twice in PBS. The washed cells were resuspended in 6M guanidine hydrochloride (5 cm^3 gm^{-1} bacteria). Following incubation (4°C, 1 hr) remaining whole bacteria and cellular debris was sedimented (35,000g, 10 min, 4°C) and the supernatant dialysed against distilled water (5°C, 96 hr). The dialysate was freeze dried and stored in a desiccator. Electrophoretic separation of constituent proteins was carried out as outlined above (2.8.1).

2.12.22 Extraction of outer membrane proteins using ethylenediamine-tetracetic acid

Broth cultured bacteria were treated using the method of Poxton & Brown (1979). Broth cultured bacteria were sedimented (35,000g, 30 min, 4°C) and washed twice in PBS. The washed pellet was resuspended in 25 cm^3 PBS containing 10 mM EDTA (20 cm^3 gm^{-1} bacteria). Following incubation (45°C, 30 min) whole cells were sedimented by centrifugation (35,000g, 10 min, 4°C) and the supernatant dialysed against volumes of 0.01 M Tris-HCl pH 7.4 containing 0.01% ($^v/v$) 2-Mercaptoethanol (x2, 5 hr). The dialysed preparation was freeze dried and stored in a desiccator. Electrophoretic separation of proteins was carried out as outlined above (2.8.1).

2.12.23 Examination of envelope proteins using crossed immuno electrophoresis (CIE)

Initially, crossed immuno-electrophoresis was employed in the analysis of serum proteins (Verbruggen, 1975). It has since become evident that CIE is the method of choice for the analysis of bacterial membranes (Owen, 1980); not only does the technique resolve the antigenic complexity of the bacterial cell with considerable clarity but antigens examined by this method retain sufficient intrinsic activity to permit functional characterisation.

CIE of V. anguillarum sphaeroplast preparations into agarose gels containing rabbit-anti-envelope antisera was carried out based on the techniques of Axelson, Kroll & Weeke (1973) and Axelson (1975) employing a LKB 2117 Multiphor horizontal electrophoresis apparatus.

Envelope preparations were dissolved in 4% $^{v}/_{v}$ Triton X-100 in 5 mM EDTA pH 8.6 to give a final protein concentration of 20 mg cm^{-3} (by Lowry et al., 1951). Protein preparations were applied (10 µl) to agarose gels (5.2 x 5.2 cm) and electrophoresed in the first dimension (15 mA, 20 hr) followed by electrophoresis into agarose containing antisera (5 mA, 20 hr). Following pressing and drying under weighted filter paper (1.5 Kg, 30 min) gels were stained with Coomassie Brilliant Blue as outlined above (2.8.1).

2.12.24 Raising of antibody in rabbits against cell envelope preparations

Raising antibody in rabbits against V. anguillarum cell envelope preparations for use with crossed immuno-electrophoresis was carried out as follows:-

To a volume of envelope preparation (2.12.20) containing 5 mg protein cm^{-3}, an equal volume of Freund's complete adjuvant was added.

Following emulsification, 50 µl aliquots were injected at multiple sites (about 30) on the back of the rabbit. After 12 days, a booster injection was given using the same envelope preparation in Incomplete adjuvant at a dose rate of 0.25 cm^3, subcutaneously near the branchial and femoral lymph nodes, and 0.25 cm^3 intramuscularly into each thigh. The booster injection was repeated 12 days later. Approximately two weeks later, and at one week intervals thereafter, rabbits were bled from the marginal ear vein until sufficient serum had accrued (2.5.3).

2.12.25 Extraction and separation of plasmid DNA

Methods used were essentially those of Guerry et al. (1974) and Meyers et al. (1976). Cleared lysates were prepared by sequential addition of 0.4 ml lysozyme (5 mg cm^{-1}, 5 min) and 0.15 cm^3 EDTA (0.25M, 5 min) at 0^oC, and 0.16 ml SDS (50 mg/ml) and 2.0 ml NaCl (3M, 4 hr) at 4^oC. After centrifugation (38,000g, 4^oC, 30 min) 0.4 ml ribonuclease (bovine pancreatic, 0.3 mg cm^{-3}, pre-heated for 10 min at 90^oC) was added and incubated (37^oC, 1 hr). Protein was precipitated by extraction with equal volumes of phenol-tris (twice) and chloroform (twice). Sodium acetate (1.0 cm^3, 1.2M) was added to the aqueous phase, and DNA precipitated by addition of 2 volumes of ethanol (-20^oC, 18 hr) centrifuged (16,000g, -10^oC, 30 min), and resuspended in 0.5 cm^3 NaCl (50 mM), EDTA (5 mM), tris (30 mM), pH 8.0. Plasmid DNA was isolated on 3 mm thick horizontal gels of 0.7% agarose in tris (89 mM), EDTA-Na_2 (2.5 mM), boric acid (8.9 mM), pH 8.0, with NaCl (3.0 M) added to the tank buffer. Ten µl of a mixture of bromophenol blue (7% $^w/_v$), glycerol (3% $^w/_v$) and SDS (7% $^w/_v$) was added to the samples (50 µl) and electrophoresis performed at constant voltage (120v) for about 3.5 hr at 10^oC. Gels were immersed in ethidium bromide (0.4 mg cm^{-3}) and plasmid DNA visualised under u.v.

light (254 nm).

These techniques were carried out by C.B. Munn and R.J. Pearcey as a part of a collaborative study and results are included in this thesis by permission.

2.12.26 Antibiotic resistance and its relationship to virulence

The possibility that V. anguillarum virulence factors may be linked to resistance/sensitivity to antibiotics was examined by determining the response of strains to a range of antibiotics (2.2.5). Both inter- and intra-strain variations were assessed.

2.12.27 Possible correlation between growth of V. anguillarum on selective and differential media and virulence

The potential use of selective and differential media as a means of expressing virulence was assessed by culturing strains on a range of media (Table 3). Initial qualitative growth was further examined for quantitative differences in the ability to grow on media not routinely used for culturing V. anguillarum.

2.12.28 Biochemical identification tests and their relationship to virulence

To examine the possibility of a relationship existing between biochemical reactions and virulence, test results (2.2.4) were assessed with respect to virulence in eels.

2.12.29 Relationship between growth rate in vitro and virulence

In order to examine the possibility of a relationship existing between growth rate in vitro and virulence, strains were cultured in TSB (25^{o}C) and the growth monitored by viable counts (2.2.6). The mean

generation time was determined by plotting time against bacterial number on logarithmic graph paper and extrapolating the exponential growth rate.

3.1.0 INTRODUCTION

To date, vibriosis has been mainly studied in dead and moribund fish from epizootics and isolated outbreaks, using histopathology and to a less extent, pathophysiology. Histopathological studies have been documented in both wild and cultured fish stocks (Håstein, 1974; Wolke, 1975; Horne et al., 1977; Richards, 1980) and certain aspects of pathophysiology have been considered (Harbell et al., 1979).

The present chapter reports the first detailed study of vibriosis in eels employing light and electron microscopy (2.9.1) to elucidate the pathogenesis of this disease. Furthermore, data concerning the behaviour of the bacterium during pathogenesis (2.9.2) and aspects of pathophysiology (2.9.3 - 2.9.8) are reported.

The anatomy of the fresh water eel seems not to have been studied in any great depth, consequently diagrammatic representations of dissected eels have been included (Fig. 4 & 5) to facilitate comprehension of disease pathology (3.2.2 and 3.2.5).

For comparative purposes, vibriosis was also studied in the elasmobranch dogfish and the edible frog.

3.2.0 THE PATHOLOGY OF VIBRIOSIS IN EELS

3.2.1 Pathology of vibriosis in eels from a natural epizootic

3.2.2 Necropsy

Moribund eels obtained from an epizootic at Hinkley Point fish farm appeared discoloured and lethargic, with skin lesions and petechial haemorrhages of body surfaces. The fins and anus were haemorrhagic and congested. On dissection (2.9.1) the liver was found to be pale, and the spleen and kidney liquefied. The gut

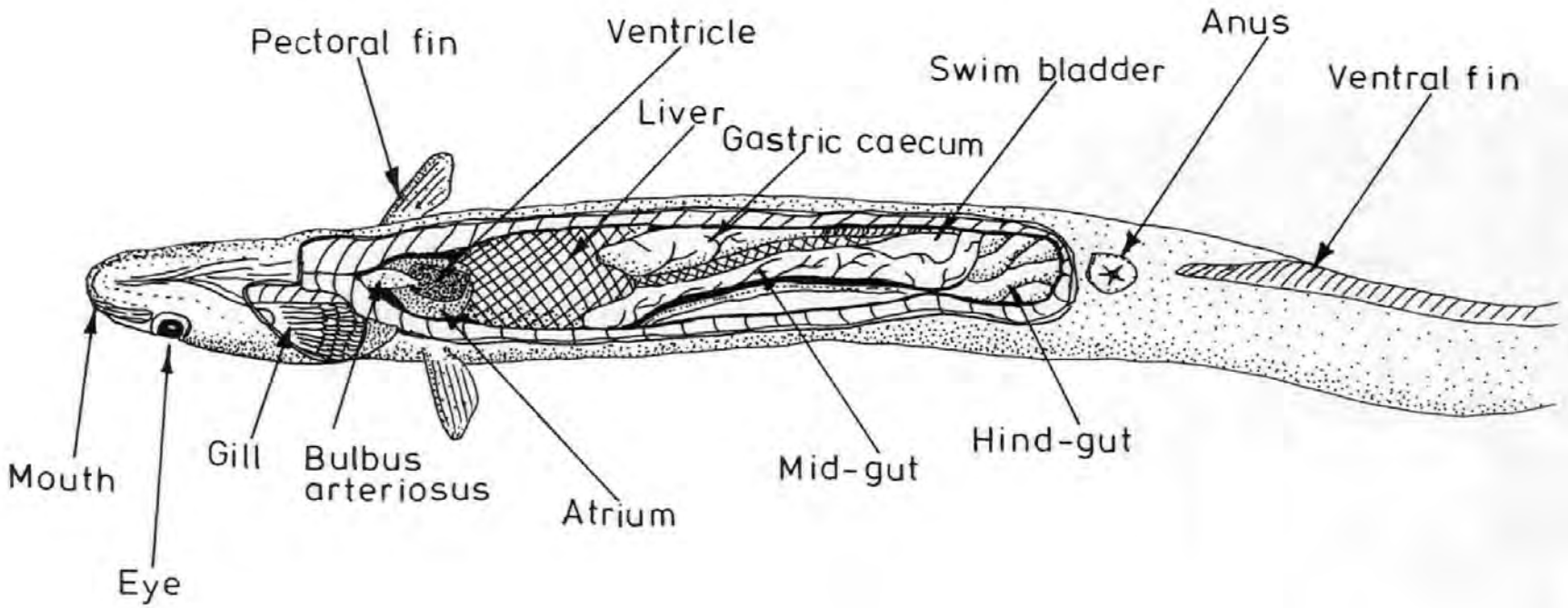


Fig 4 - Ventral view of eel with wall of abdomen and operculum removed.

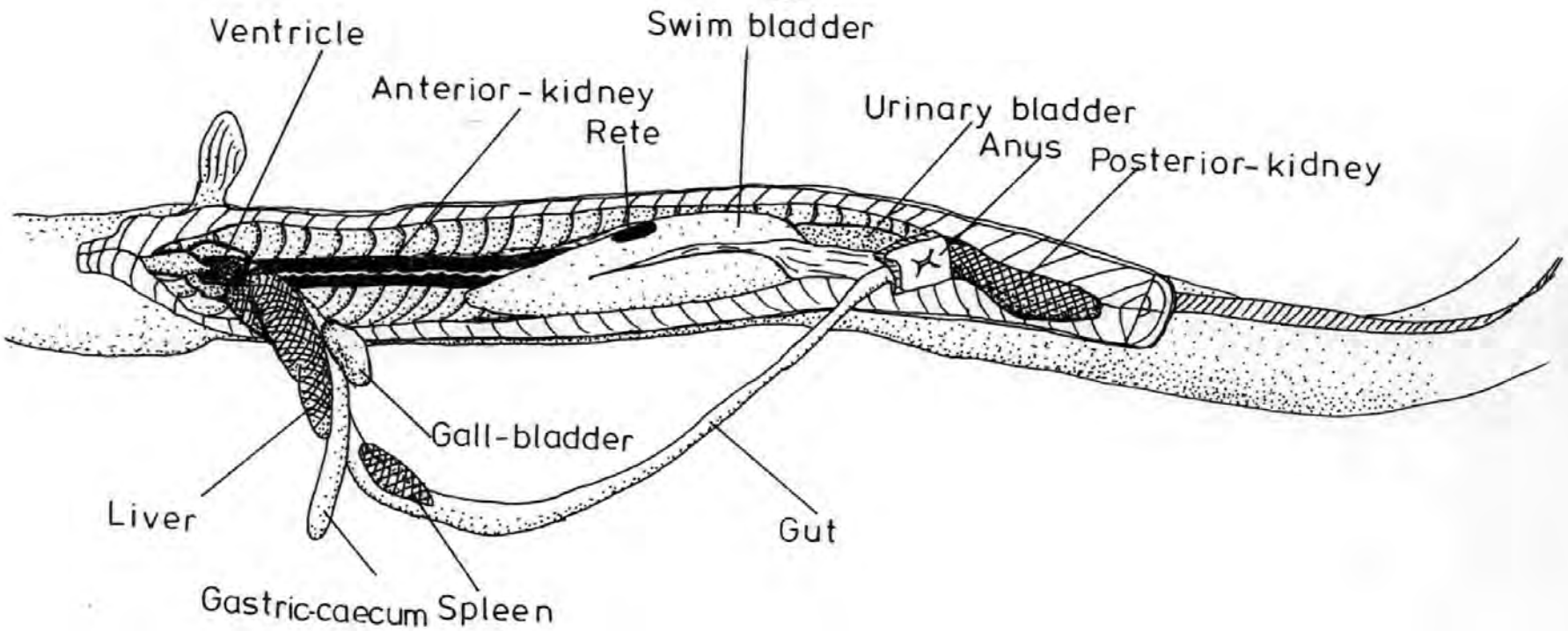


Fig 5 - Ventral view of eel showing dissection of alimentary canal

appeared red and distended with fluid, and vasodilation of mesenteric blood vessels was evident. Removal of opercula revealed the gills to be pale with localised areas of filament haemorrhage. Observations of infected plasma showed signs of haemolysis.

3.2.3 Histopathology of naturally infected eels

Light microscopy of spleen sections demonstrated severe haemorrhaging and cellular necrosis of parenchymal cells with an apparent reduction in melanomacrophage centres. Infected liver was found to be haemorrhagic with vasodilation of hepatic blood vessels. Both infected and control liver sections were characterised by numerous intracellular vacuoles. Kidney sections revealed haemorrhage and cell necrosis of parenchymal cells, accompanied by detachment of the renal tubular epithelia at the basement membrane and reduction in melanomacrophage pigment.

The heart of diseased fish was particularly affected, with extensive necrosis of the myocardium, in both ventricle and atrium, and haemorrhaging of the bulbus arteriosus. The alimentary canal contained no food or faeces and was greatly distended with fluid. Although the mucosal epithelia appeared intact, infiltration of the submucosa and connective tissue by red and white cells was evident. Observations of gill sections revealed extensive necrosis and haemorrhaging of gill filaments and secondary lamellae with vasodilation of afferent and efferent blood vessels.

3.2.4 Pathology of experimental vibriosis in eels

3.2.5 Necropsy

Following infection of eels with vibriosis (ip injection, 10^8 broth cultured bacteria), death occurred approximately 24 hr post-

inoculation. Early signs of the disease included minute petechial haemorrhages of the skin and discoloration of the injection site. As the pathogenesis progressed petechial haemorrhages became more pronounced with haemorrhaging of the fins, and anus. Moribund eels became blotchy and discoloured with a noticeable increase in respiratory rate; death followed characteristic spasmodic and erratic swimming behaviour.

External manifestations of vibriosis in eels are illustrated (Plate 1a & b). Close examination of the injection site revealed this location to be haemorrhaged and swollen, with grey necrotic areas at the centre. The anus was swollen and red, discharging a thick mucoid exudate, which bacteriology revealed to contain large numbers of V. anguillarum. The buccal cavity was frequently affected, having petechiae on the walls of the mouth and tongue.

Occasionally eels died with deep ulcerous lesions exposing the under-lying musculature (Plate 2). Exophthalmus, although occasionally observed (Plate 3a) was not a characteristic feature of vibriosis in eels. Plasma from diseased fish contained fragments of erythrocyte debris suggesting in vivo haemolysis, while control blood samples had normal clear, green plasma. Blood smears (Plate 3b) and tissue prints, stained with Giemsa, demonstrated large numbers of systemic bacteria and a noticeable increase in leucocytes in the tissues.

Post-mortem examination of eels revealed extensive haemorrhaging of the peritoneal wall, the alimentary canal appearing red and exhibiting vasodilation of the mesenteric blood vessels; the gut was invariably distended with fluid (Plate 4a & b). The liver was discoloured and there were minute petechiae on the surface. The kidney and spleen appeared enlarged, haemorrhagic and liquefied, becoming fragmented during dissection; loss of melanomacrophage material in

infected spleens was particularly noticeable (Plate 4c & d). Removal of the operculum revealed petechial haemorrhages on the operculum wall and congestion of gill filaments.

3.2.6 Histopathology

Histological preparations of skin and muscle from the injection site (Plate 5) showed detachment of the cuticle, with haemorrhaging and leucocytic infiltration of the epidermis, stratum spongiosum and stratum compactum. Myofibrils of the underlying myotomes appeared necrotic with generalised oedema. Cell-free culture supernatant did not cause these manifestations.

Sections of whole eel established that infected spleen tissues (Plate 6a) were haemorrhaged, with cellular necrosis and loss of tissue integrity, such that red and white pulp areas observed in control sections (Plate 6b) became indistinguishable from one another. A reduction in melanomacrophage centres was also apparent. Spleen tissues examined by electron microscopy confirmed light microscopic observations. Numerous bacteria were present in the spleen which were characterised by an electron-lucent zone surrounding the bacterial cells (Plate 7a & 7b). The normal white and red pulp areas (Plate 8a & 8b) again appeared to be destroyed during pathogenesis.

Wax sections of infected anterior and posterior kidney (Plate 9a) revealed haemorrhaging of kidney parenchymal cells with detachment of renal tubular epithelium into the lumen of the tubule. Higher magnification (Plate 9d) showed that the epithelium detached at the basement membrane. There appeared to be little change in the presence of melanomacrophage centres when compared to control sections (Plate 9b & 9c). Electron microscopy further revealed extensive haemorrhaging and cellular necrosis of kidney tubules (Plate 10a). The presence of

a large number of bacteria in this organ was again characterised by the electron-lucent zone observed in the spleen. Observations of infected renal tubules (Plate 10b) further demonstrated epithelial detachment at the basement membrane.

Light microscopy of infected liver sections (Plate 11a) demonstrated the presence of numerous erythrocytes in the intercellular spaces between hepatic cells, a feature not observed in control sections (Plate 11b). Electron microscopy revealed extensive necrosis of liver parenchymal cells with some damage of cell nuclei, and large numbers of intracellular bacteria (Plate 12a). Control tissues (Plate 12b) contained large quantities of glycogen, however this appeared to be broken down during pathogenesis (Plates 12a, 13a and 13b). Rough and smooth endoplasmic reticulum and lipid droplets remained intact during pathogenesis (Plates 12 and 13). Hepatic blood vessels (Plate 13b) contained large numbers of bacteria which apparently left the circulation and invaded adjacent tissues. Invasion of tissues by circulating bacteria was also observed in other tissues.

The alimentary canal of infected eels was particularly affected during pathogenesis. Vibriosis was characterised by infiltration of the submucosa and underlying connective tissue by large numbers of erythrocytes and leucocytes with detachment and sloughing of mucosal epithelia into the lumen of the gut (Plate 14a). Histochemical staining of diseased and control sections using periodic acid Schiff (PAS) demonstrated features including mucous cells and epithelia, facilitating observation of mucosal detachment.

Mucosal surfaces of the oesophagus and gastric caecum were not consistently affected during pathogenesis, however when it occurred epithelial detachment was characterised by fragmentation of the mucosa (Plate 14b and 15). On occasions, the gastric caecum mucosa

of diseased eels was not detached but contained numerous necrotic pits extending to the submucosa (Plate 16a) a feature not encountered in control eels (Plate 16b).

The intestine of infected fish was characterised by extensive sloughing of the gut mucosa (Plate 17a). In contrast to the gastric caecum, sheels of intestinal mucosa were detached; detachment occurring at the basement membrane (Plate 17c).

Examination of whole eel sections showed haemorrhaging of the spinal cord (Plate 18a) when compared to control tissues (Plate 18b). These sections further revealed haemorrhaging of the pancreas, gonads, swimbladder and wall of the pneumatic duct, and distension and haemorrhaging of intestinal blood vessels.

Light microscopy of diseased heart tissues failed to reveal any gross histopathological manifestations; however electron microscopy demonstrated foci of bacteria in the myocardium, with localised necrosis of cardiac tissue (Plate 19a).

Examination of gill sections showed the filament and lamella blood vessels as distended and congested; however no pathological features were apparent. Observation of gill tissues by electron microscopy revealed systemic bacteria invading and colonising adjacent gill tissues (Plate 19b).

Electron microscopical observation of skin revealed numerous foci of bacteria (Plate 19c). Histological examination of the anal exudate indicated that this mucoid material consisted largely of epithelial mucosa. Features such as mucous cells and microvilli (Plate 20a) identified the origin of these tissues by comparison with control tissues (Plate 20b). Resin sections of the exudate showed that the sloughed mucosa remained intact (Plate 20c).

3.2.7 Experimental vibriosis in non-anguillid species

The elasmobranch, Scyliorhinus canicula was found to be susceptible to vibriosis following routine infection. Fish died approximately 24 hr post-inoculation, without any major signs of disease although the ventral surfaces and anus appeared slightly red. Internally, the viscera appeared very red and haemorrhagic with large amounts of fluid in the gut. Histopathology revealed necrosis of the liver and spleen with an absence of gut mucosal detachment. Light microscopy of blood wet mounts revealed large numbers of non-motile bacteria in haemolysed blood. Concurrent bacteriology identified the causative organism as V. anguillarum.

Inoculation of the edible frog demonstrated this amphibian to be susceptible to vibriosis. Frogs died about 22 hr post-inoculation with slight haemorrhaging of the hind legs. Dissection and histopathology revealed severe haemorrhaging of liver, heart and gut (Plate 21a) however, the gut mucosa remained intact (Plate 21b).

3.2.8 Invasion of fish tissues by V. anguillarum following experimental infection

The dissemination of bacteria within the host was monitored following intramuscular (im) injection with broth-cultured V. anguillarum (2.9.2). Blood samples removed at regular intervals and streaked on to blood agar plates, revealed the presence of bacteria in the blood within 1 hr of inoculation. Enumeration of bacteria in salient tissues (2.2.6) during pathogenesis revealed large numbers of bacteria in all tissues examined (Table 10, p.133), by 6 hr post-inoculation. Bacterial numbers increased in all tissues during pathogenesis with particularly large numbers present in the spleen. Bacterial isolates from this experiment were identified as V. anguillarum.

3.2.9 Location of phagocytic areas in the eel

Eels injected intravenously with 0.1 cm^3 of a colloidal carbon suspension were killed and fixed for histology 2 days post-injection. Wax sections revealed that carbon particles were removed from the circulation mainly by spleen and kidney macrophages producing large black deposits readily observed in tissue sections (Plate 22). Gill macrophages and circulatory monocytes also contained small amounts of carbon.

3.2.10 The status of host iron following challenge with V. anguillarum

Eels experimentally challenged with V. anguillarum were found by histochemistry (2.7.2) to have deposits of haemosiderin in liver sections (Plate 23a), a feature not encountered in control tissues. Injected eels were found to have liver iron deposits 3 hr post-inoculation.

Fish dying with vibriosis following experimental challenge had haemosiderin deposits in the liver, at death; however eels from the epizootic at Hinkley Point were lacking this pigment.

Haemosiderin deposits were found amongst the melanomacrophages of both treated and control spleen and kidney (Plate 23b & c).

3.3.0 PATHOPHYSIOLOGY OF VIBRIOSIS

3.3.1 Effect of vibriosis on the eel electrocardiograph (ECG)

Electrocardiographical experiments were carried out on three occasions using strain UNH 569 (2.9.3). Death occurred approximately 10 hr post-inoculation, eels displaying typical manifestations of vibriosis, including petechial haemorrhages of the skin and fins, and internal haemorrhaging of the organs. In addition, tissues in the

close vicinity of the electrodes were particularly affected. The ECG data from all three experiments were similar in the overall response, however only one set of data is given here.

Following implantation of electrodes, the cardiac cycle (Fig. 6) stabilized with a heart rate of approximately 60 beats min^{-1}, a ventricular depolarisation voltage of about 250 µV and an arterial voltage of about 20 µV. The P-T wave (Fig. 6) had a duration of 0.57 ± 0.03 sec, with a T-P duration of 0.48 ± 0.14 sec (Fig. 7).

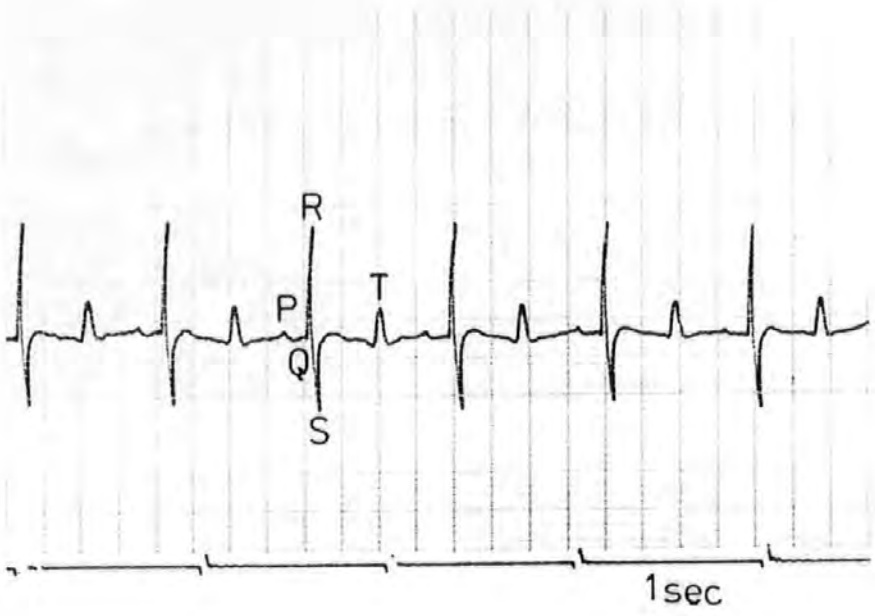
Infection instigated a gradual increase in the ventricular and atrial depolarisation voltage reaching a maximum at 320 µV and 60 µV respectively, 3 hr post-inoculation. The peaks in the P and RS waves were associated with an increase in heart rate to a maximum of 75 beats per minute, a shortening of the P-T cycle and a reduction in the heart recovery time T-P. From this point in time the ventricular voltage gradually decreased with a concomitant increase in the P-T and T-P durations until death of the animal some 3½ hr later. The atrial voltage continued at 60 µV until very near death which was preceeded by a sudden drop in the atrial voltage.

3.3.2 Effect of experimental vibriosis on the plasma unsaturated iron-binding capacity

The response of eels to infection with *V. anguillarum*, with respect to iron status was investigated (2.9.4). Plasma removed from eels 3 hr post-inoculation, showed that fish receiving viable bacteria had an increased UIBC (1.93 µg) as compared to control eels (1.07 µg).

3.3.3 Ionic status of fish with vibriosis

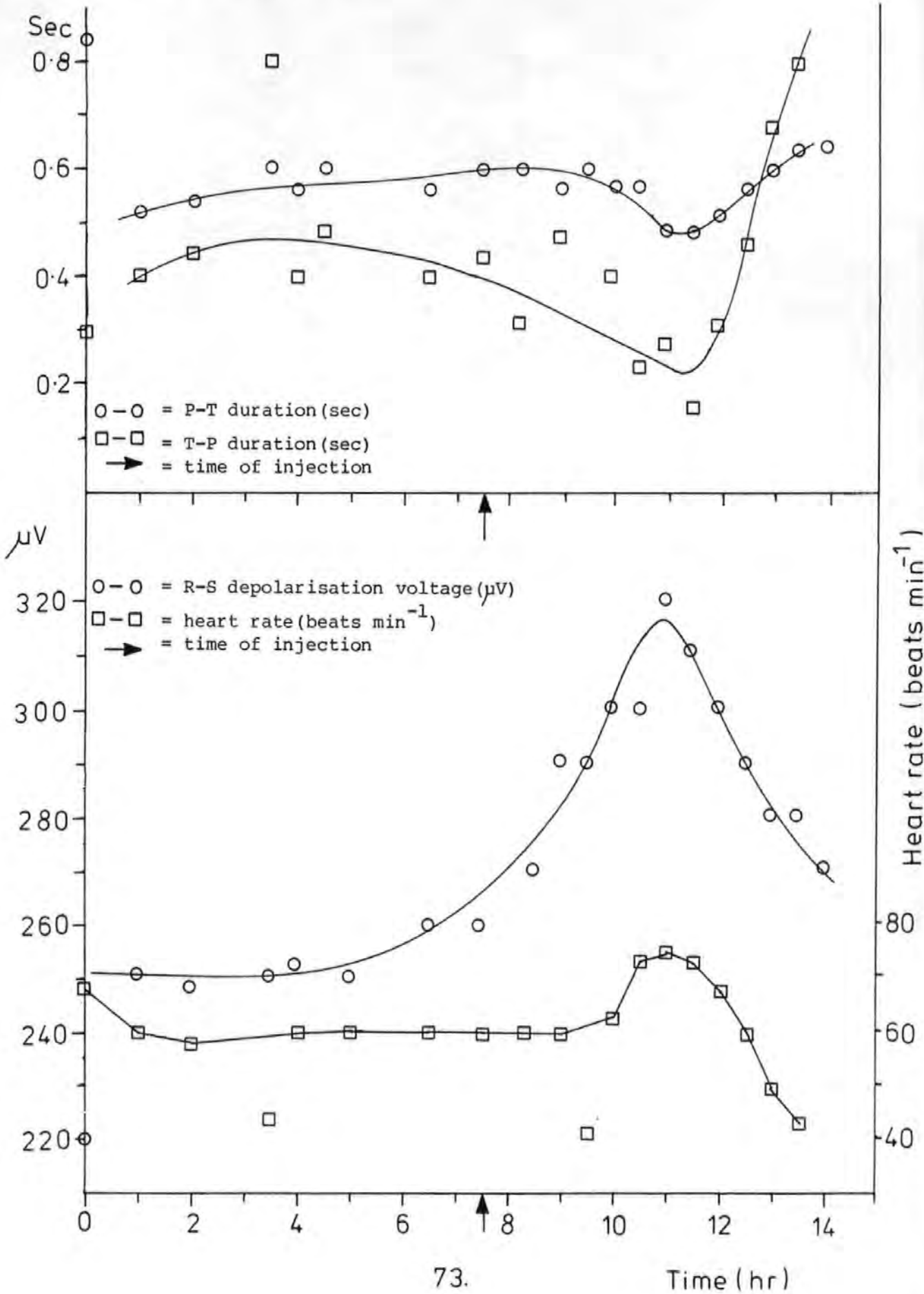
The intestinal fluid observed in fish with vibriosis, was removed from larger specimens of eel and dogfish by means of a sterile syringe.



Peak	Relationship to cardiac cycle
P	Depolarisation of atrium.
QRS	Depolarisation of ventricle.
T	Repolarisation of myocardium

Fig 6 Electrocardiograph of eel illustrating the normal cardiac cycle and the relationship to myocardial contraction.

Fig 7 The effect of vibriosis on the eel electrocardiogram during pathogenesis.



Chemical analysis of the fluid (2.9.8) revealed higher levels of sodium, potassium and chloride than detected in normal fish plasma (Table 6).

Pathogenesis studies showed the presence of fluid in the gut by 12 hr post-inoculation, containing slightly less of the ions as compared to moribund eels (Table 6).

3.3.4 Analysis of plasma proteins from eels with vibriosis

Separation of plasma proteins, from eels with vibriosis, by polyacrylamide electrophoresis (2.9.5) showed a general reduction in protein content as compared to control plasma (Plate 24a). High molecular weight globulins appeared unaffected however, protein bands coinciding with human albumin and haptoglobulin appeared to be reduced in diseased samples. Protein separation in the presence of SDS (Plate 24b) showed an absence of proteins of molecular weight 26,500, 43,000 and 47,000 in diseased material, whilst having an extra protein of 39,000 daltons, not present in control plasma.

3.4.0 DISCUSSION

3.4.1 Histopathology

European eels and non-anguillid species were successfully infected with vibriosis, death occurring approximately 20-30 hr post-inoculation. Experimental infection of fish with vibriosis has been reported by other workers; Muroga & Egusa (1967) infected ayu (*Plecoglossus altivelis*) and Japanese eels (*Anguilla japonica*) by intravascular injection, ayu dying in thirty days and eels dying in two days.

Gross pathological manifestations of vibriosis encountered during this study correlated with those described in eels (Bruun & Heiberg,

Table 6 Ionic content of gut fluid from fish with vibriosis as compared to control sera.

Ion	Ionic content mEq dm^{-3}		
	12 hr post-inoculation	Moribund	Control sera
Eel			
Sodium	146.7 ± 4.5	176.6 ± 17.6	161.2 ± 4.9
Potassium	4.8 ± 0.2	6.2 ± 0.1	4.1 ± 0.3
Chloride	38.0 ± 8.5	213.7 ± 23.0	nd
Dogfish			
Sodium	nd	280.0	nd*
Potassium	nd	10.3	nd
Chloride	nd	554.0	nd

nd* = not determined

1932; McCarthy, 1976; Miyagaki et al., 1977) and other fish species (Bullock et al., 1971; Anderson & Conroy, 1970), bearing some similarity to those of furunculosis caused by Aeromonas salmonicida, Pseudomonas reptilivora infections in mummichogs (Li et al., 1978) and shigellosis in mammals (Watkins, 1960). The spasmodic swimming behaviour encountered in moribund eels after experimental infection, was in contrast to the lethargy observed in eels dying during the natural outbreak at Hinkley Point, and that reported by Bruun & Heiberg (1932). Haemorrhaging of the spinal cord observed by histopathology, may have interfered with nervous control of muscle function causing the observed erratic swimming. Corneal opacity and exophthalmus was not a characteristic feature of vibriosis in eels however, this observation seems to be prevalent in this disease in turbot and salmonids (Ross et al., 1968; Håstein & Holt, 1972; Hodgins et al., 1977; Horne et al., 1977; Richards, 1980). Ulceration occasionally observed in moribund eels appears to be characteristic of vibriosis in trout and salmon (Roberts & Shepherd, 1974) further suggesting that certain external manifestations of vibriosis are species specific.

The absence of food and faeces in the gut of eels dying during the Hinkley Point outbreak suggested the disease to be associated with a loss of appetite, a feature reported elsewhere (Anderson & Conroy, 1970; Harbell, 1976). In these fish a feature of both infected and control eels was the presence of large vacuoles in liver sections. The eel diet was known to be high in oils hence these liver vacuoles were thought to be lipid deposits, the lipid having been removed during histological preparation by dehydrating and clearing agents, leaving translucent spaces.

Pathogenesis of vibriosis in experimentally infected eels

commenced with an initial colonisation of the circulation where V. anguillarum became established. The circulation provided transport for bacteria to all tissues of the body where establishment and multiplication lead to large numbers of tissue bacteria, the spleen and kidney containing particularly large numbers of bacteria.

Throughout histopathological studies at electron microscopical levels, it was apparent that bacteria present in tissues were surrounded by a translucent zone, the significance of which was examined in Chapter 6.

Histopathological studies were greatly facilitated by using whole fish sections (TS) which has not been reported previously. Tissues usually difficult to excise by dissection such as swim-bladder, pancreas and gonads were easily observed in situ. Furthermore, tissues such as spleen which become liquefied during pathogenesis (Roberts, 1978) and easily damaged during dissection could readily be observed by this method.

Haemorrhaging and liquefaction of the spleen tissues appeared to be a feature of both experimental and natural vibriosis in eels. The large numbers of bacteria present in this tissue would seem to be as a consequence of bacterial phagocytosis by splenic macrophages. Although the occurrence of phagocytosis in vivo was not proven, phagocytic ability of this tissue was demonstrated and intracellular bacteria were observed in liver and spleen by electron microscopy (Plate 25a & b).

Haemorrhaging and necrosis was also observed in the kidney, with detachment of renal tubular epithelia occurring early in pathogenesis. As with the spleen, tissue damage was probably attributable to retention of bacteria. The importance of inflammatory suppuration in disease of these tissues was obscured by indigenous leucocytes, however tissue prints demonstrated a generalised leucocytosis which may account for

the reduction in circulating white cells observed by Harbell et al. (1979). In mammalian pathology, leucocytosis can be readily produced by the injection of bacterial products and/or polypeptides derived from damaged host plasma and tissues (Muir, 1964).

During pathogenesis, a loss of melanomacrophage centres was observed in spleen and kidney, a feature reported elsewhere (Håstein, 1974; Richards, 1980). Whether these pigments were actually broken down during pathogenesis or mobilised as a consequence of bacterial invasion remains unclear. Agius (1980) showed the pigmented elements of fish melanomacrophage centres to contain melanin and lipofuscins, the former having been reported as having bacteriostatic properties (Crossley, 1979) and implicated in host defence mechanisms (Edelstein, 1971).

The haemorrhaging of infected liver tissues shown here and reported elsewhere (Bruun & Heiberg, 1932; Novotny et al., 1975; Anderson & Conroy, 1970) was shown to be accompanied by extensive cellular necrosis as revealed by electron microscopy. Although it was found that large numbers of bacteria developed in the liver during pathogenesis, the very high numbers demonstrated in the spleen were not attained. Keppie et al. (1955) described large numbers of tissue bacteria present in guinea-pigs dying with anthrax. The reason for the high bacterial numbers required to cause death was thought to be because toxin production per bacterial cell was low and therefore large numbers of bacteria were required to produce lethal quantities of the toxin.

The anal exudate encountered during pathological studies on infected fish and reported elsewhere (Saito et al., 1975; Harbell, 1976) was found to contain portions of the intestinal mucosa. The cause of this desquamation remains unclear however, the inflammation

observed in the submucosa and connective tissue could be involved in suppurative necrosis of these tissues. It has been shown that V. anguillarum produces at least one haemolysin (Chapter 6) and that haemolysins can be cytolytic to cells other than erythrocytes (Bernheimer, 1974; Alouf, 1977). It seems likely that bacterial products including enzymes produced by V. anguillarum (Chapter 6) could be directly involved in cell necrosis. Mason & Balis (1980) have shown that bacterial exo- and endo-toxins damage the endothelium in mammalian blood vessels, however the effect of these components on fish tissues has still to be elucidated. Reasons for the loss of gut epithelia in 'sheets' as opposed to fragmentation, as observed in the gastric caecum remain unclear, however structural differences would affect both inflammatory suppuration and bacterial insult. To elucidate the role of inflammation in gut desquamation, attempts were made to suppress the immune response of eels prior to infection using a known mammalian immuno-suppressant : Cyclophosphamide (Bellanti, 1971; Roitt, 1977). Inoculated fish died with typical manifestations of vibriosis including intestinal desquamation. Infiltration of leucocytes into the submucosa suggested that cyclophosphamide was unable to suppress the eel immune response; Maisse & Dorson (1976) showed cyclophosphamide to have no effect on immunisation of trout with chloroform-killed A. salmonicida since the immune response in fish is still obscure further research would have to be carried out to determine the exact effect of cyclophosphamide.

The high levels of electrolytes present in the gut lumen may explain the reduction of host plasma sodium, potassium and chloride ions experienced by salmon with vibriosis (Harbell et al., 1979). Excessive loss of ions is important in the pathogenesis of cholera caused by V. cholera and other diarrhoeal diseases of humans

(Craig, 1976). Sodium and potassium in particular are key electrolytes in numerous physiological functions and their loss would presumably have important effects on the maintenance of physiological equilibrium.

In eels experimentally infected with vibriosis, wax sections of heart appeared only slightly affected during pathogenesis. However, this organ was shown to be severely affected during a natural outbreak, and appears to be affected in other species (Horne et al., 1977; Roberts, 1978; Richards, 1980). Ferguson (1975) showed the presence of phagocytic cells in the atrium of plaice and Horne et al. (1977) suggested these cells may have retained V. anguillarum in the atria of diseased turbot causing extensive myonecrosis. It seems unlikely that this would be the cause of myocardial damage in eels as phagocytic cells were not found in the eel heart.

The determination of vibriosis in the elasmobranch dogfish has not been previously reported. The loss of bacterial motility in the blood of dogfish was investigated by placing broth cultured bacteria in a urea gradient; it was found that reduction in motility became apparent at concentrations approaching that of isotonic dogfish saline (3g urea 100 cm^{-3}, Young, 1933), and severely retarded growth when cultured anaerobically. Although urea may have had an effect on the organism in vivo other compounds such as trimethylamine oxide (TMO) may have been involved in ceasation of motility. As with infected teleosts, vibriosis in dogfish was also characterised by a loss of electrolytes into the gut lumen.

Vibriosis produced in the frog was reported by Canestrini (1893) who also infected newts with this disease. However an interesting feature of vibriosis in the frog was lack of gut desquamation. Why amphibia, shown to be susceptible to vibriosis, do not lose their

gut epithelia and to what extent manifestations of vibriosis change with phylogenetic development, remains unclear.

3.4.2 Pathophysiology

The haemolysis observed in fish dying with vibriosis was probably the cause of the reduction in erythrocyte number in chinook salmon (Cardwell & Smith, 1971) winter flounder (Levin et al., 1972) and coho salmon (Harbell et al., 1979). Although haemodilution (Harbell et al., 1979) and/or blood loss via lesions (Levin et al., 1972) may have been responsible for the observed reduction in blood cell counts, the vibrio toxin would seem a more probable cause.

Studies on the effect of vibriosis on the fish heart have not been reported to date. Furthermore the fish heart has not been researched to any great extent; however existing data (Kisch, 1948; Oets, 1950) shows that although the fish heart is physiologically different from the mammalian hearts, the ECG appears to be similar. The present research showed the eel ECG to change dramatically during pathogenesis of vibriosis. Initial increases in heart rate and 'strength' of myocardial contraction were thought to be as a consequence of bacterial insult with some stress occurring due to the presence of the heart electrodes. Haemolysis frequently observed in infected fish could have been partially or wholly responsible for the increase in heart rate due to the reduced oxygen-carrying capacity of the blood. A decrease in circulatory oxygen has been shown to produce tachycardia in fish (Kisch, 1948). Following a peak in ventricular depolarisation and contraction rate the cardiac cycle gradually slowed down until death of the fish, and there are numerous possible reasons for this. As with the mammalian heart, the pace-maker initiates the wave of excitation that passes through the Perkinji

fibres leading to contraction (Hoar, 1966); bacterial invasion, as demonstrated by electron microscopy, could damage these tissues involved in impulse transmission. The observed haemolysis could also have affected heart function due to disturbance of the potassium-sodium balance as was observed by Halbert (1971) following injection of mice with the haemolytic toxin - streptolysin.

In eels, the pacemaker in the atrium has been shown to govern the spread of excitation through the other pacemakers (Grodzinski, 1954), the severe atrial necrosis shown here in naturally infected eels and reported elsewhere (Horne et al., 1977; Roberts, 1978; Richards, 1980) could impair function of the atrial pacemaker and the general function of the heart.

It is important to realise that the eel heart is physiologically different from those of other fish species (Mott, 1951), and the ECG can be directly affected by environmental temperature (Grodzinski, 1954), and location of electrodes (Kisch, 1948). Precautions were taken to minimise these factors.

The observed reduction of plasma proteins following separation by SDS and non-SDS polyacrylamide electrophoresis, in particular of albumin, was also encountered by Harbell et al. (1979). They showed a significant decrease in plasma proteins, including albumin in coho salmon with vibriosis. Reduction of human haptoglobulin-like protein observed in diseased plasma was of particular interest as in human pathology, haptoglobulins bind free haemoglobin and play an important role in the degradation of haemoglobin to bile pigments. They are destroyed during this process and since their rate of synthesis is slow the concentration of haptoglobulin falls in haemolytic conditions (Gray, 1968).

The reduction in the β_2 (glycoprotein) portion of plasma protein

profiles reported by Harbell et al., 1979) was not apparent during these studies. Loss of plasma proteins was reported by Hunn (1964) who found decreases in plasma protein in brook trout (Salvelinus fontinalis) with corynebacterial disease. He suggested that the loss of protein was due to kidney damage and a reduction in protein synthesis as a consequence of liver damage. The importance of this has to be clarified in vibriosis in the eel. The 39,000 dalton polypeptide observed in infected plasma was thought to be a breakdown product of higher molecular weight proteins or of bacterial origin, as the major component of the vibrio outer membrane contains proteins of a similar size (Chapter 6).

When studying the histopathology of fish disease it is important to be aware of histological changes which occur as a consequence of environmental influences and parasites, unrelated to the disease being studied. In the eel, physiological processes associated with the natural life-cycle of this catadromous species alter the structure of some tissues. In salinity adaptation it has been shown that the oesophagus (Hirano & Mayer-Gostan, 1976; Yamamoto & Hirano, 1978) and kidney (Oliveraeu & Oliveraeu, 1977) are modified. Also various blood parameters differ between the silver and yellow form of this species (Larsson & Fänge, 1969; Johansson et al., 1974; Lidman et al., 1979).

The freshwater eel is invariably infected by trypanosomes (Plate 3b) and intestinal Acanthocephela which could also have an effect on histological observations. During the present study (4.2.8) locally caught, offshore fish were examined for inherent bacteria. These fish were found to be infected with liver and gut parasites which could be misleading when observed during histopathological studies on fish with bacterial infections.

Plate 1a) External manifestations of vibriosis in the eel demonstrating anal exudate(arrowed) and petechial haemorrhages on the ventral surface (Mag x 1)

Plate 1b) Haemorrhaging and congestion of the pectoral fin(p) (Mag x 3).

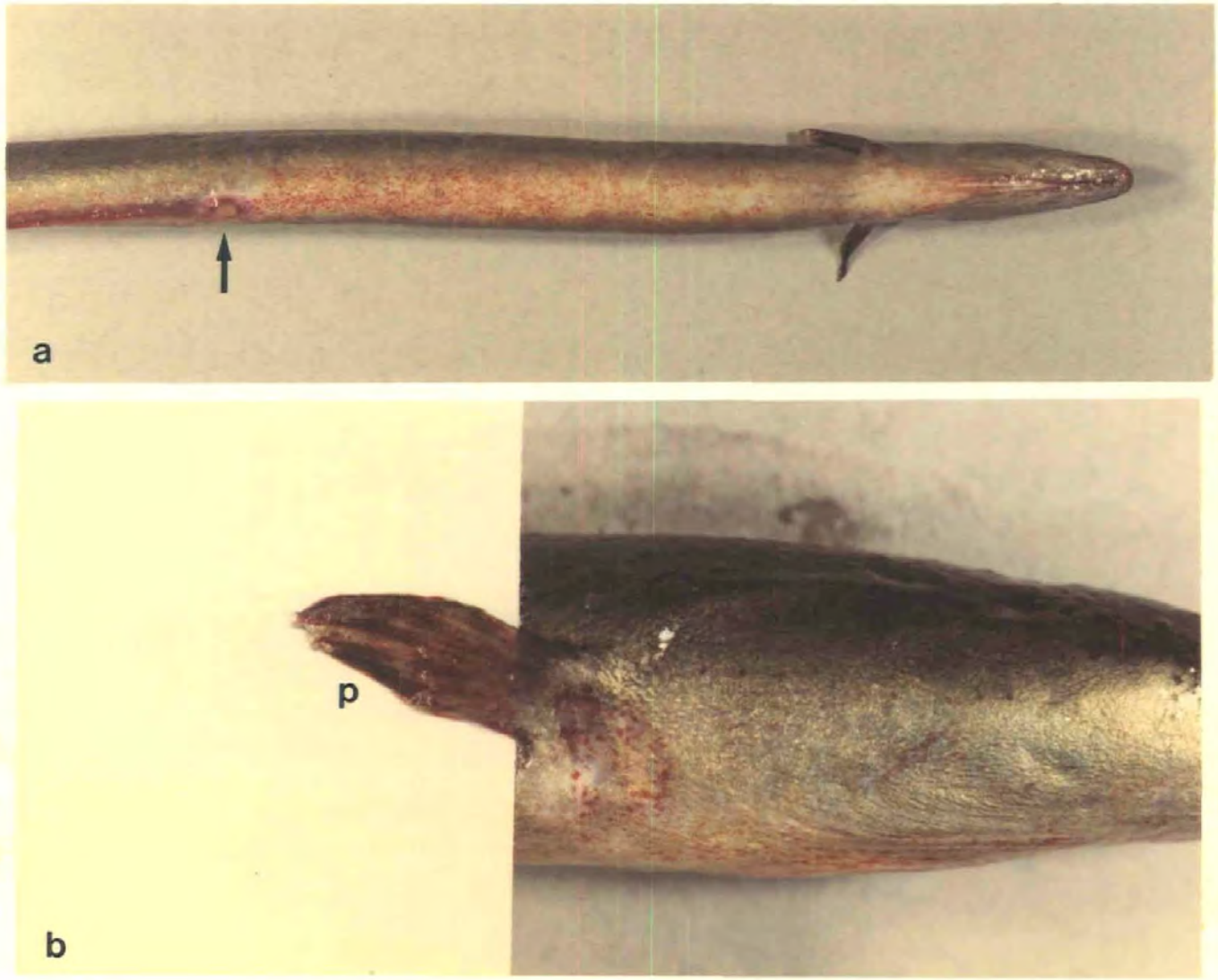
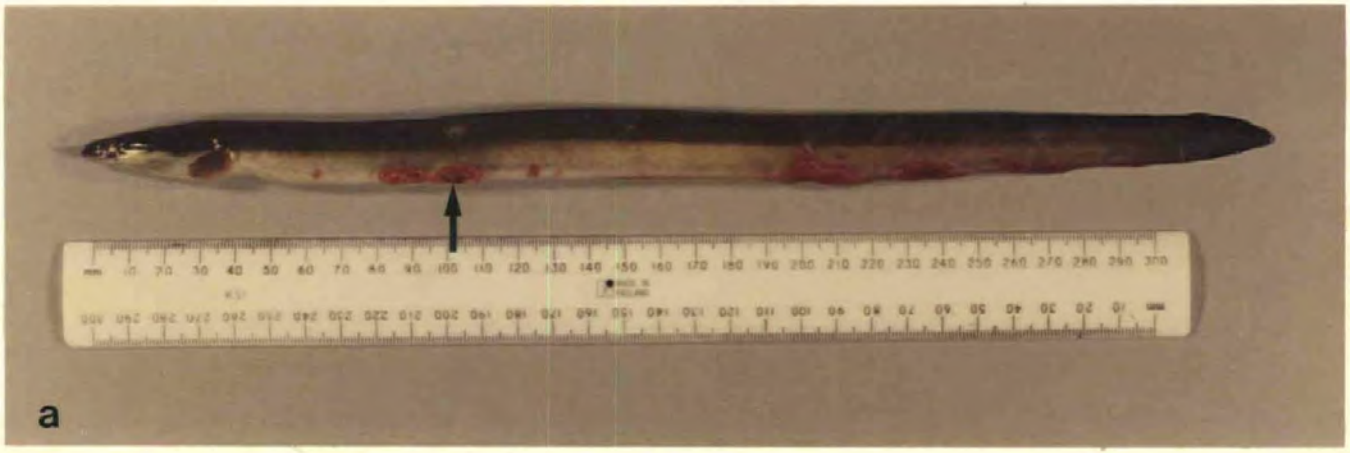
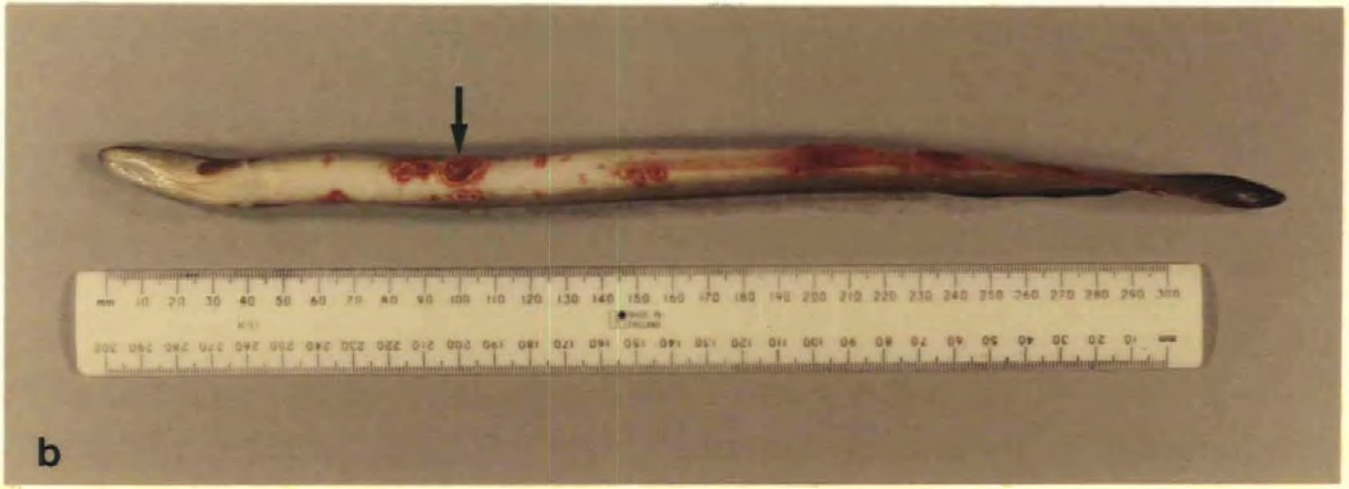

a
p
b

Plate 2 External manifestations of vibriosis in the eel demonstrating ulcerous lesions(arrowed)(Mag x ½).



a



b

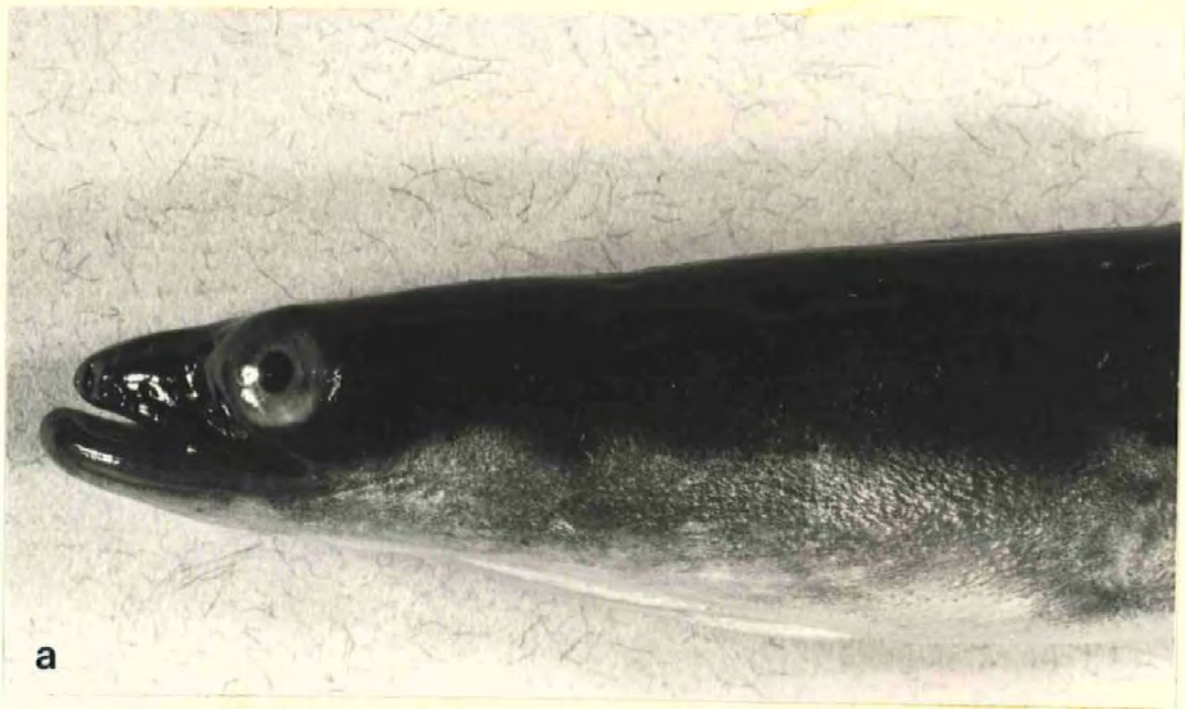
Plate 3 a) Exophthalmia in eel with vibriosis (Mag x 3).

Plate 3 b) Blood smear from infected eel demonstrating systemic bacteria(arrowed)(Giemsa, x 549,bar=10μm).

l=leucocyte

e=erythrocyte

t=*Trypanosoma* spp.


a

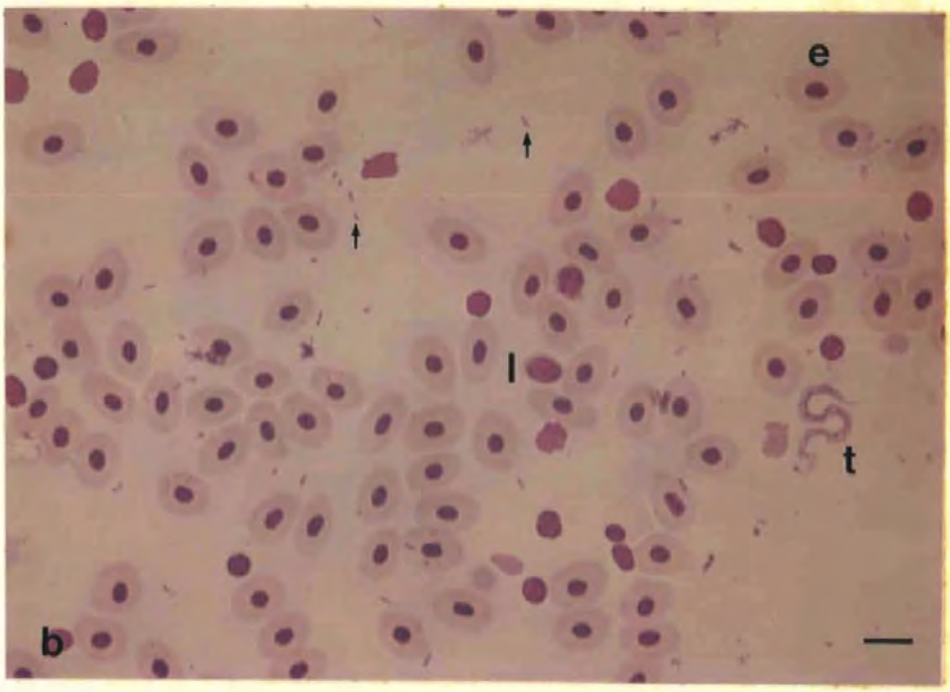

e
l
t
b

Plate 4a) Internal manifestations of vibriosis in the eel showing haemorrhaging of the heart, liver, gut and mesenteries (arrowed) (Mag x 1).

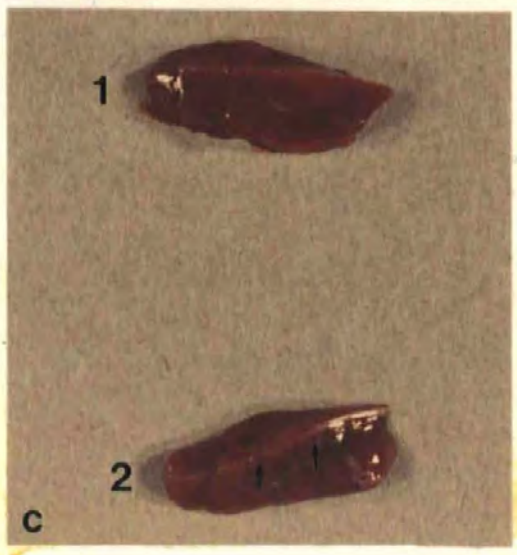
Plate 4b) Dissection of control eel demonstrating normal viscera (Mag x $^{7}/_{10}$).

Plate 4c)
Dissected spleen from infected eel (1) showing loss of melanomacrophage centres and tissue necrosis as compared to control spleen (2) (Mag x 3). arrow=melanomacrophage centres.

Plate 4 d)
Dissected posterior kidney from infected (1) eel showing haemorrhaging and liquefaction as compared to control kidney (2) (Mag x 3).


a


b


1
2
c


1
2
d

Plate 5 Light micrograph of injection site from infected eel demonstrating cuticular detachment (arrowed) from the epithelium(e),haemorrhaging of the Stratum spongiosum(Ss) and Stratum compactum(Sc) with necrosis of the underlying musculature(m) (Mallory's triple stain, x 39,bar=250μm).

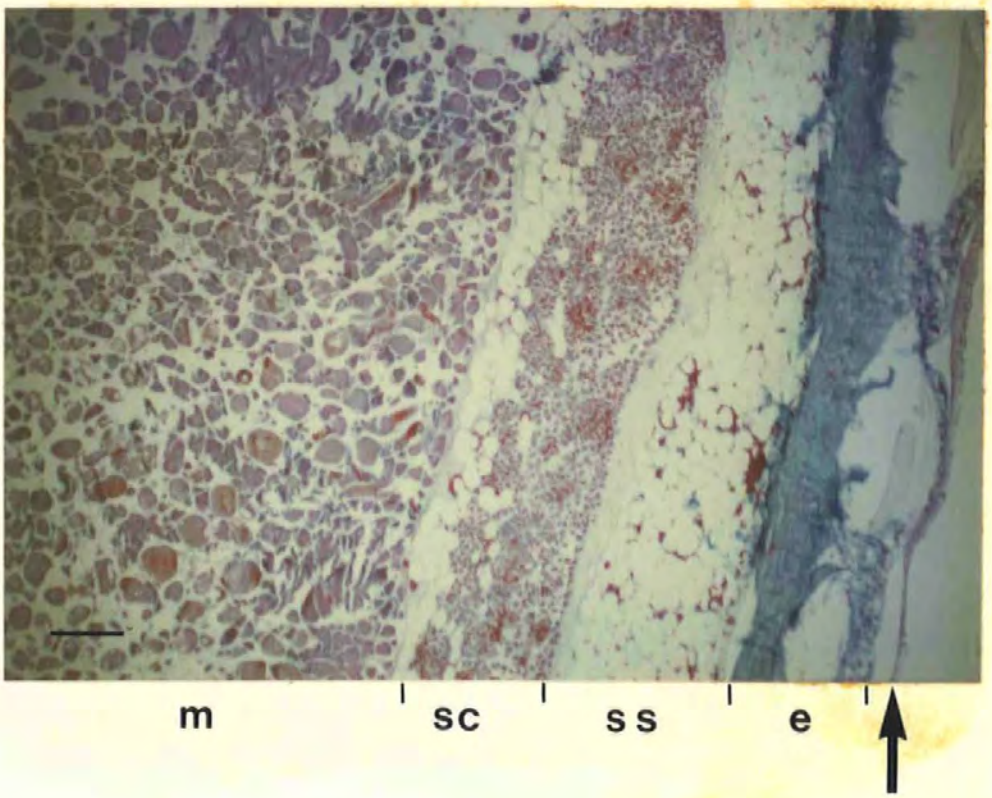

m
sc
s s
e

Plate 6a) Light micrograph of infected eel spleen showing extensive necrosis and reduction of melano-macrophage centres (mmc) (Mallory's triple stain, x 238, bar=50μm).

Plate 6b) Light micrograph of control eel spleen showing normal tissue appearance (Mallory's triple stain, x 238, bar=50μm).
rp=red pulp
wp=white
pulp
mmc=melanomacrophage centre.

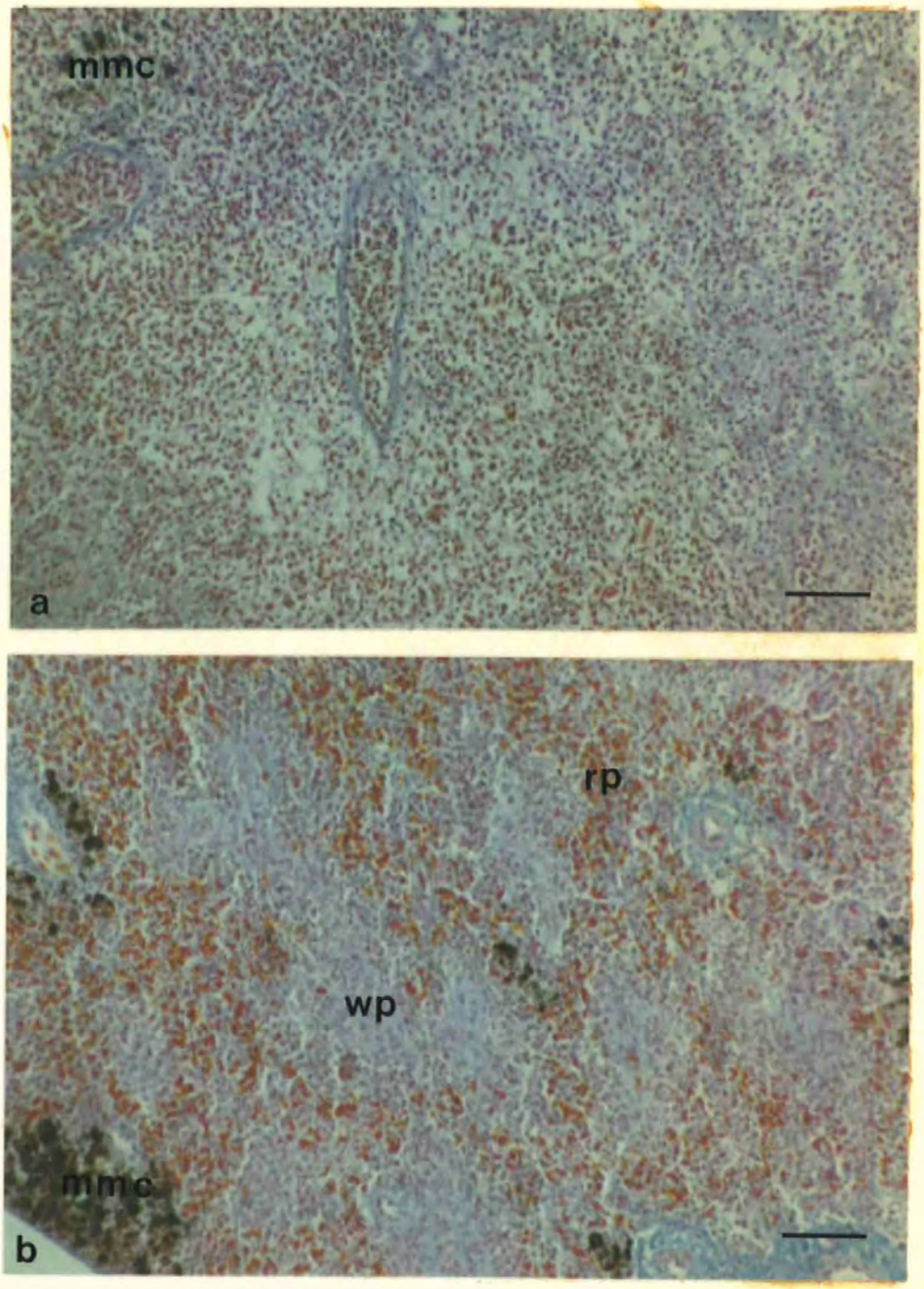

mmc
a
rp
wp
mmc
b

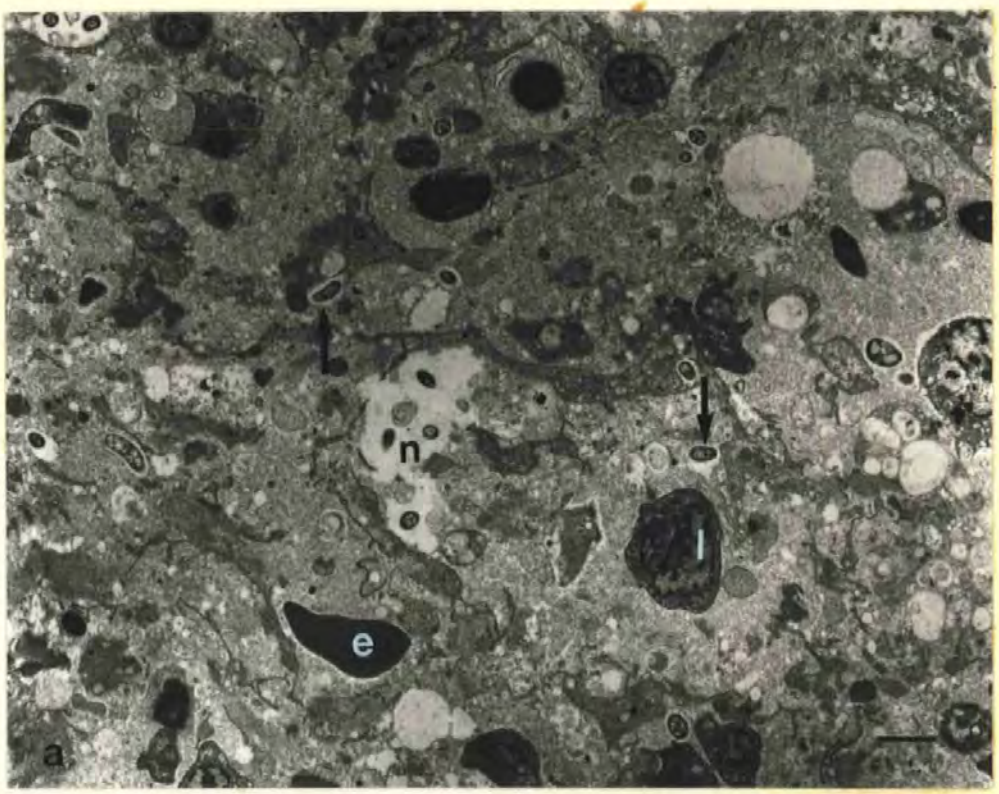
Plate 7 Electron micrographs of infected eel spleen demonstrating cellular necrosis(n) and bacteria(arrowed).

l=leucocyte

e=erythrocyte

a) x 3300,bar=2μm

b) x 2760,bar=2μm


n
l
e
a

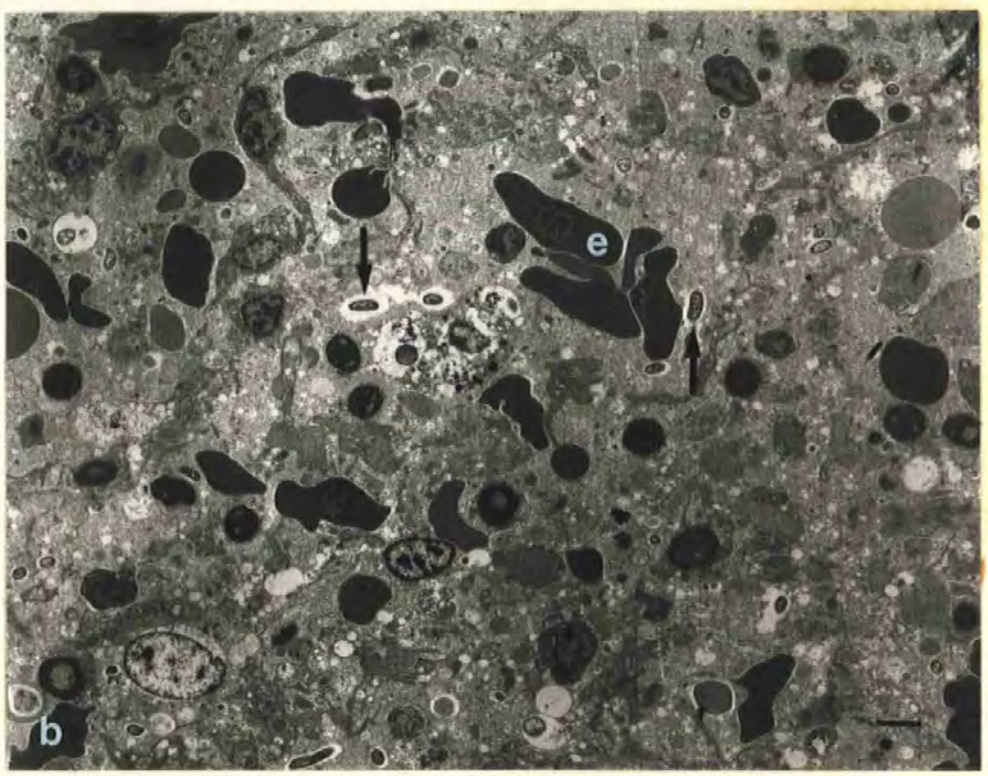
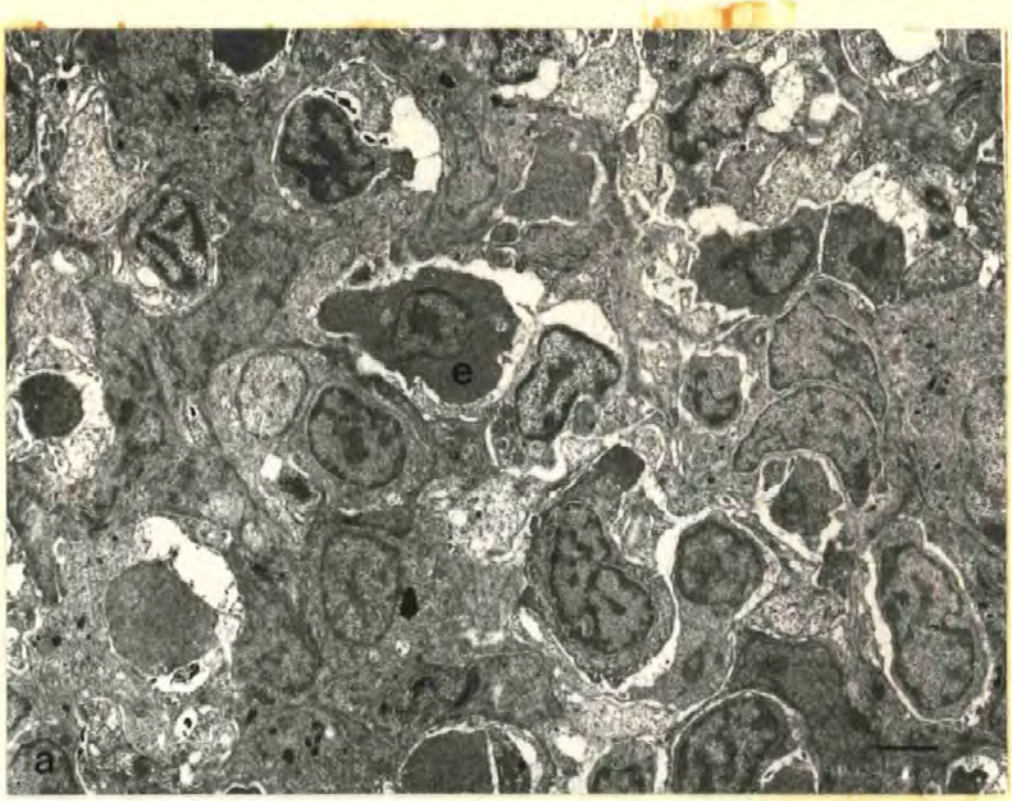

e
b

Plate 8 Electron micrographs of control eel spleen showing a)white pulp and b) red pulp(x4950,bar=2μm).

l=leucocyte

e=erythrocyte

mmc=melanomacrophage centre.


e
a

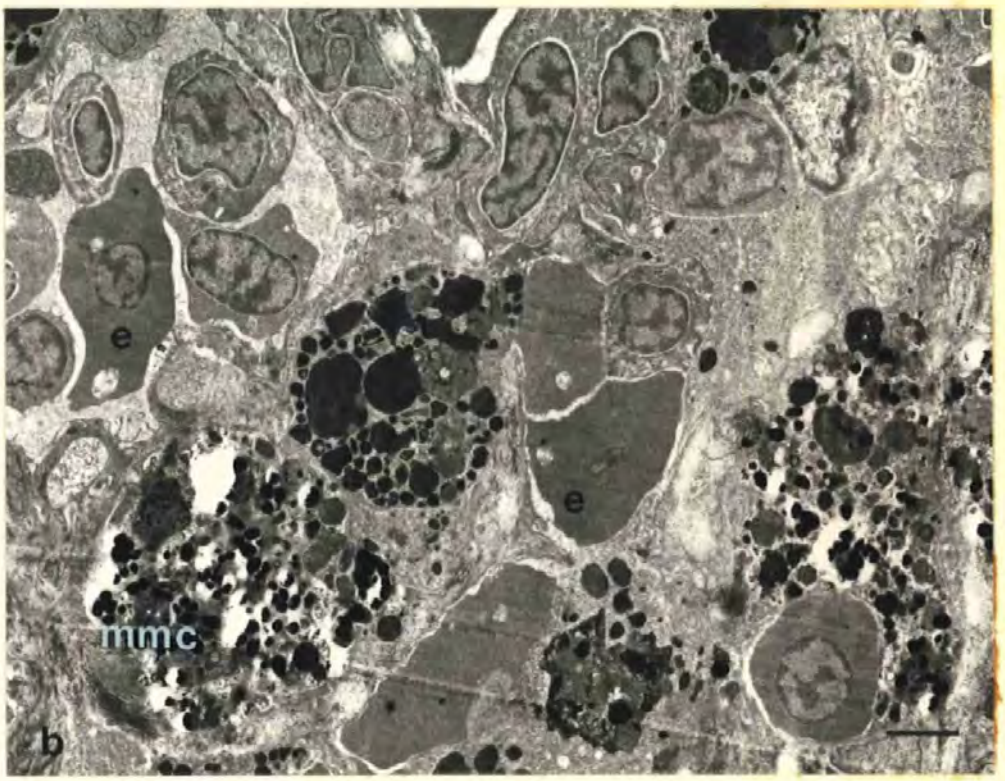
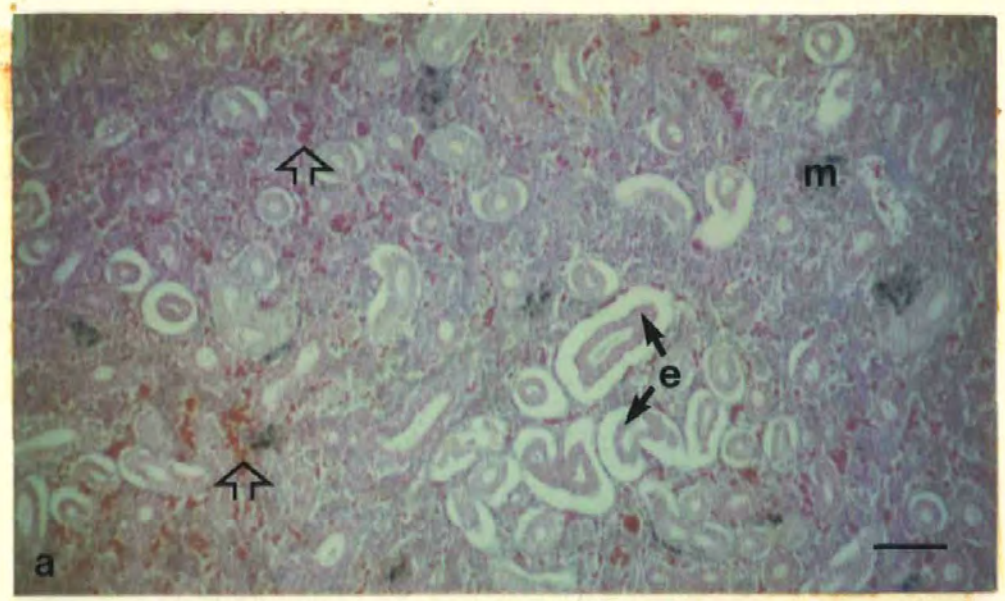

e
e
mmc
b

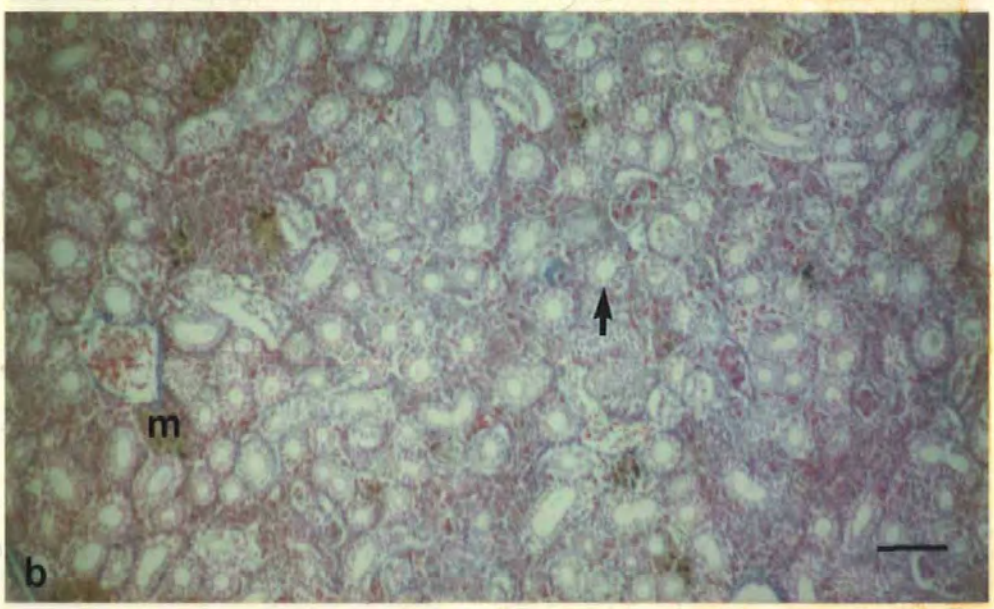
Plate 9a) Light micrograph of infected eel kidney demonstrating renal tubular epithelium detachment(e) and haemorrhaging (Mallory's triple stain, x 94,bar=100μm).

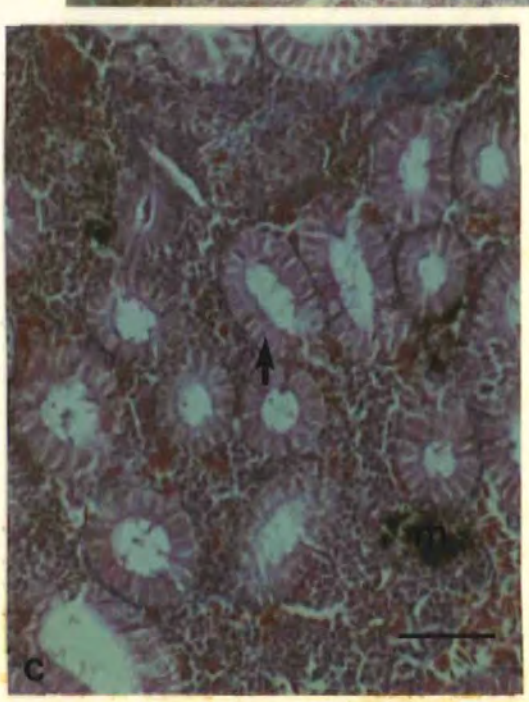
Plate 9b) Light micrograph of control eel kidney indicating renal tubular epithelia(arrowed)(Mallory's triple stain, x 94, bar=100μm).
m=melanin

Plate 9c) Light micrograph of control kidney indicating renal tubular epithelia(arrowed)(Mallory's triple stain, x 238,bar=50μm). m=melanin.

Plate 9d) Light micrograph of infected kidney demonstrating loss of renal epithelia(arrowed) at the basement membrane(bm) (Mallory's triple stain, x 238,bar=50μm).


m
e
a


m
b


c

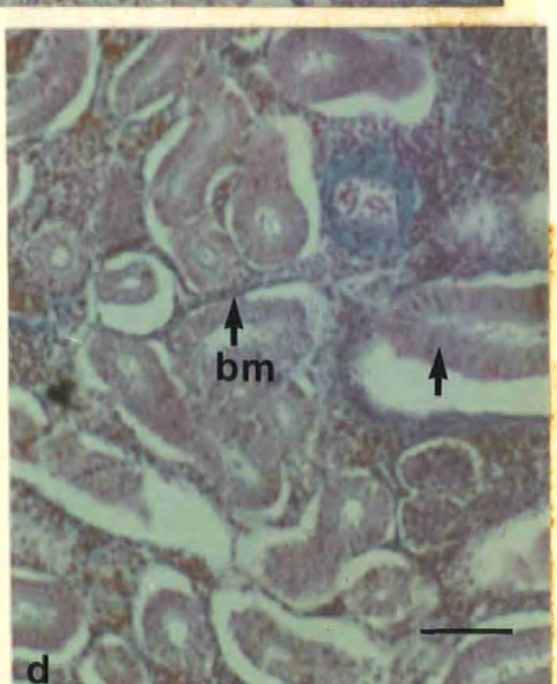

bm
d

Plate 10 a) Electron micrograph of infected eel kidney demonstrating cellular necrosis(n) and tissue bacteria(arrowed) (x 2769,bar=2μm).

e=erythrocyte.

Plate 10 b) Electron micrograph of infected eel kidney tubule showing detachment of renal epithelium(ep) at the basement membrane(bm) (x 4050,bar=2μm).

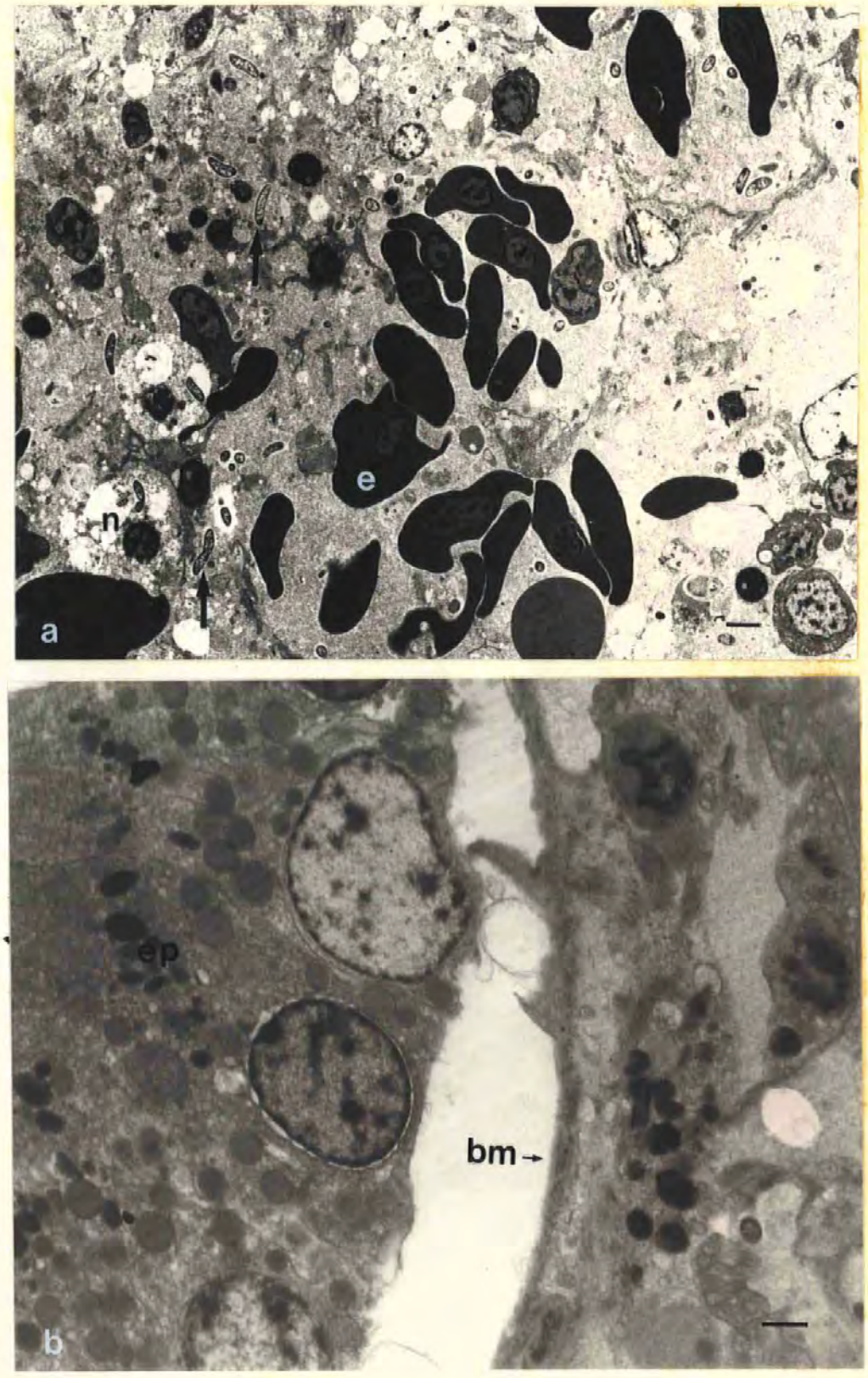
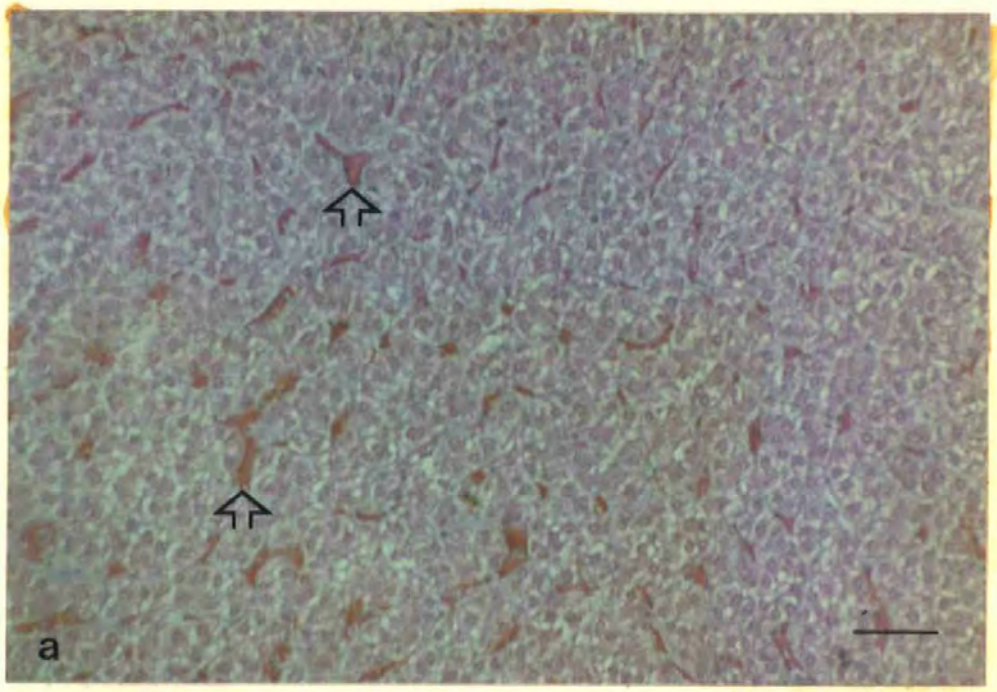

e
n
a
ep
bm
b

Plate 11 a) Light micrograph demonstrating haemorrhaging(⇨) of infected eel liver(Mallory's triple stain, x 238,bar=50μm).

Plate 11 b) Light micrograph of control liver (Mallory's triple stain, x 238,bar=50μm).


a

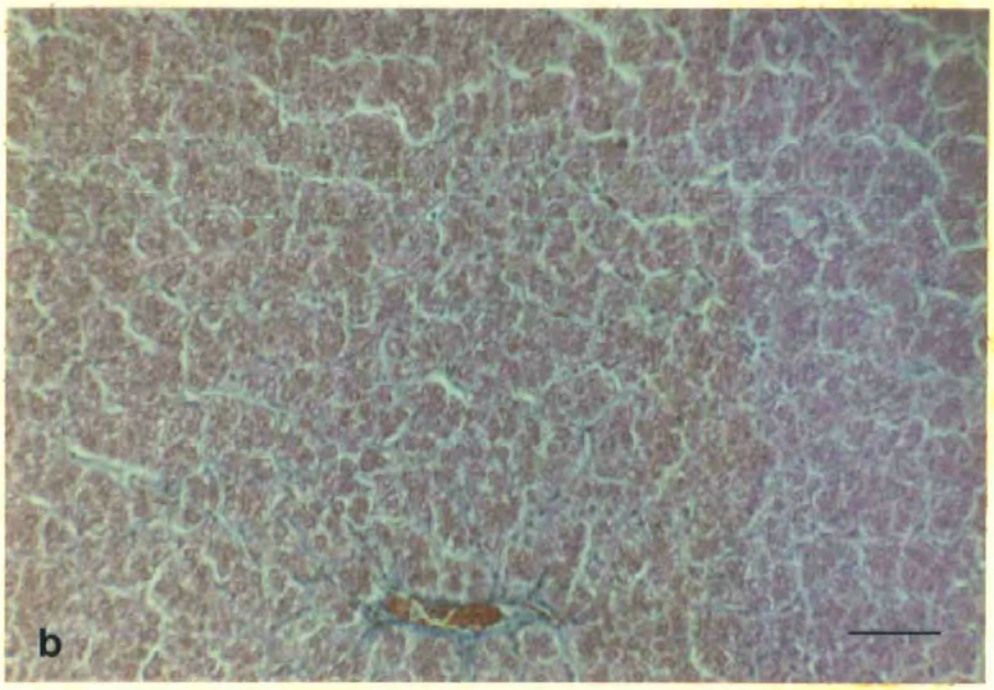

b

Plate 12 a) Electron micrograph of infected liver demonstrating tissue necrosis(n) and bacteria(arrowed) (x 4950,bar=2μm).

N=nucleus

e=erythrocyte

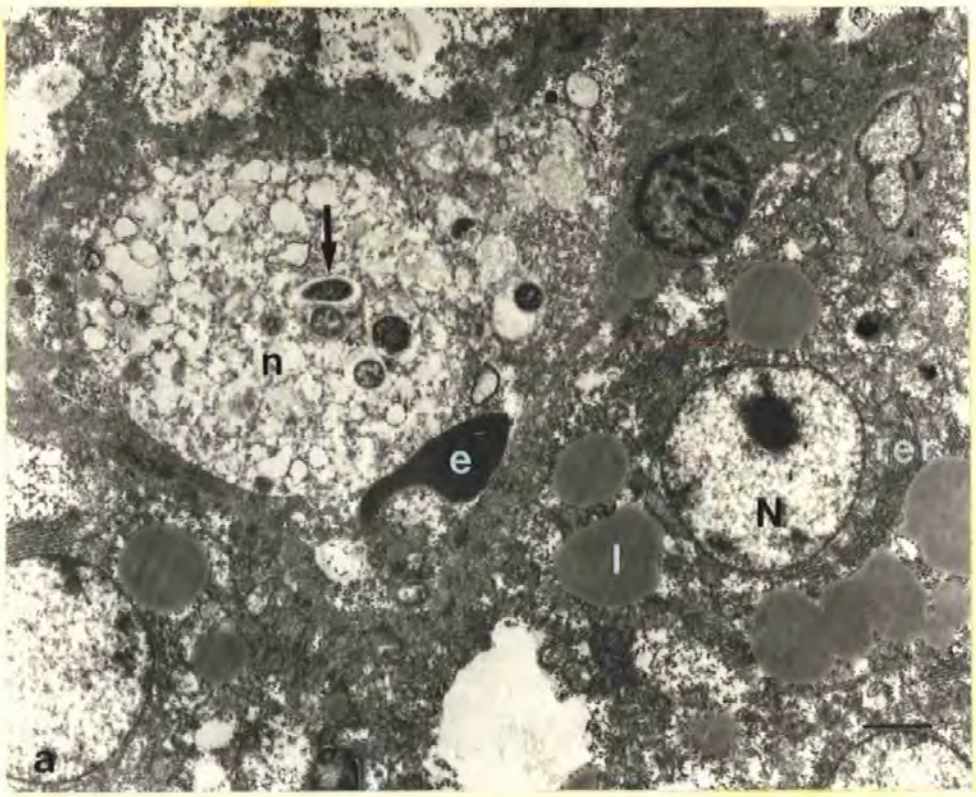
rer=rough endoplasmic reticulum

l=lipid.

Plate 12 b) Electron micrograph of control eel liver tissue showing glycogen deposits(gly),(x 9450,bar=1μm).

N=nucleus

rer= rough endoplasmic reticulum.


n
e
N
rer
l
a

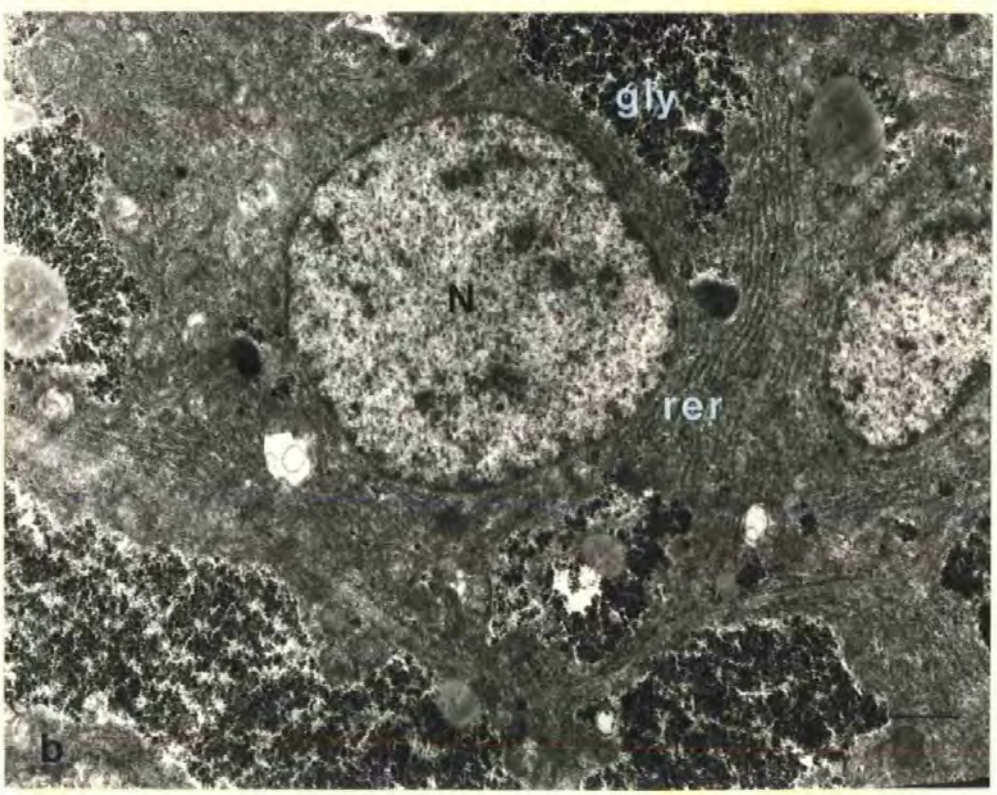

gly
N
rer
b

Plate 13 a) Electron micrograph of infected eel liver showing cellular necrosis with apparent absence of lipid break-down(l), (x 4950,bar=2μm).

e=erythrocyte

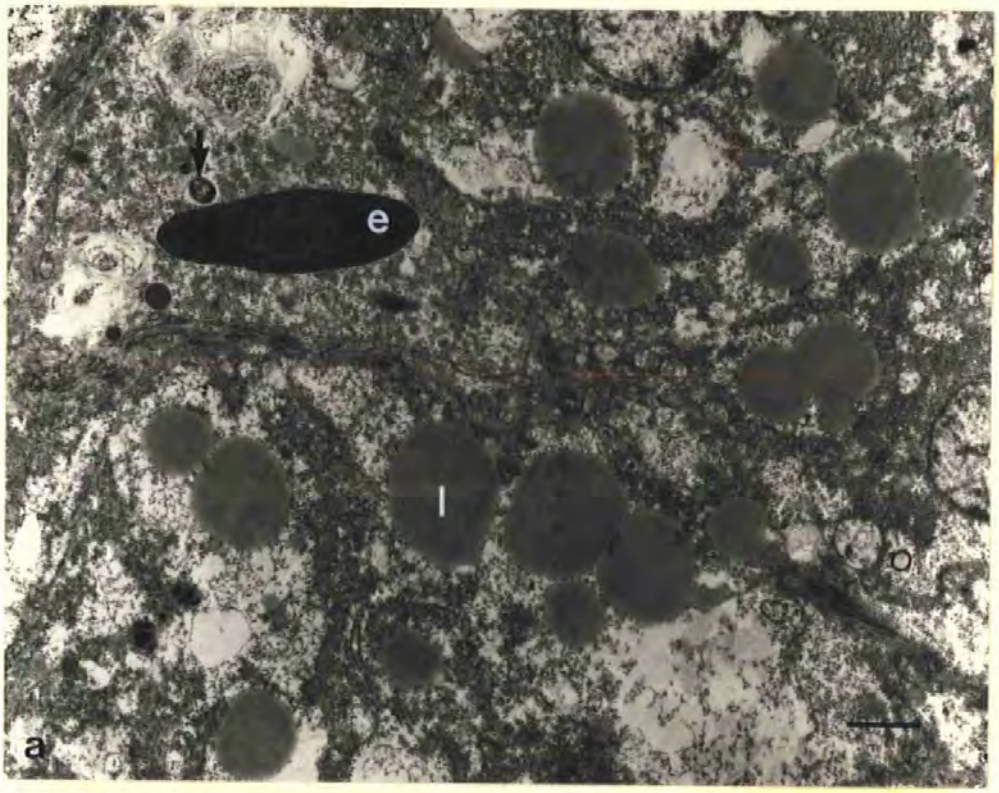
bacteria (arrowed).

Plate 13 b) Electron micrograph of infected eel liver demonstrating systemic bacteria(arrowed) invading adjacent tissues from hepatic blood vessel(bv),(x 4050,bar=2μm).

N=nucleus

rer= rough endoplasmic reticulum

l=lipid.


e
l
a

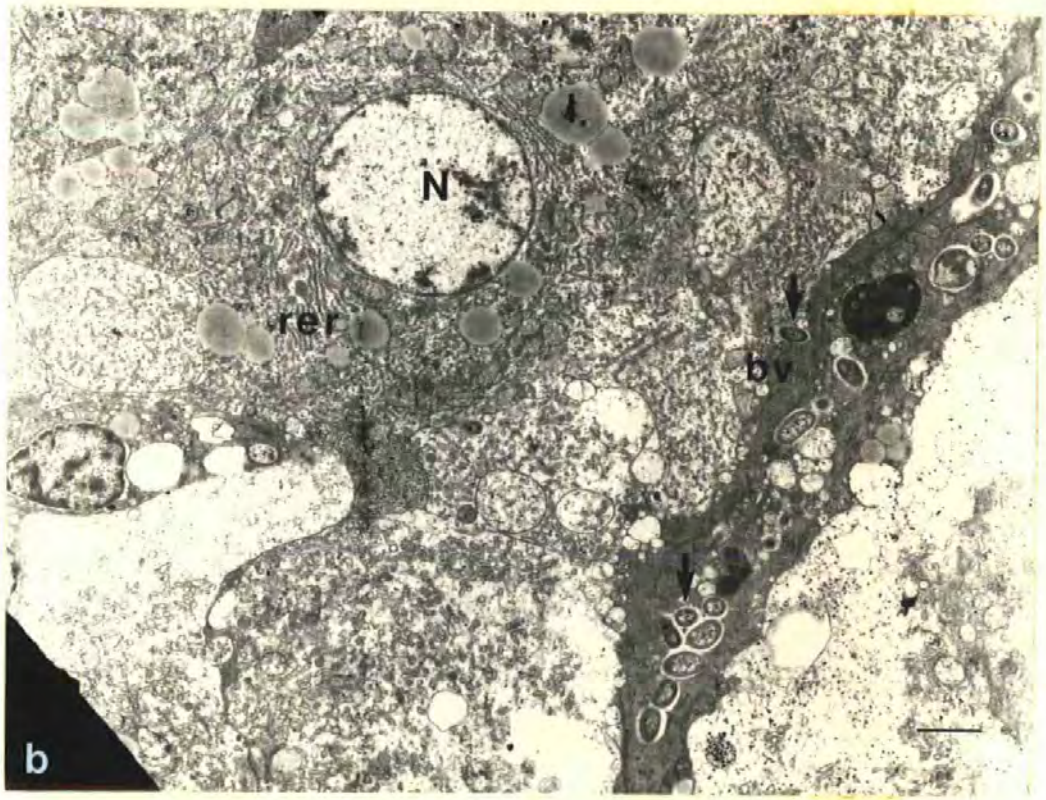
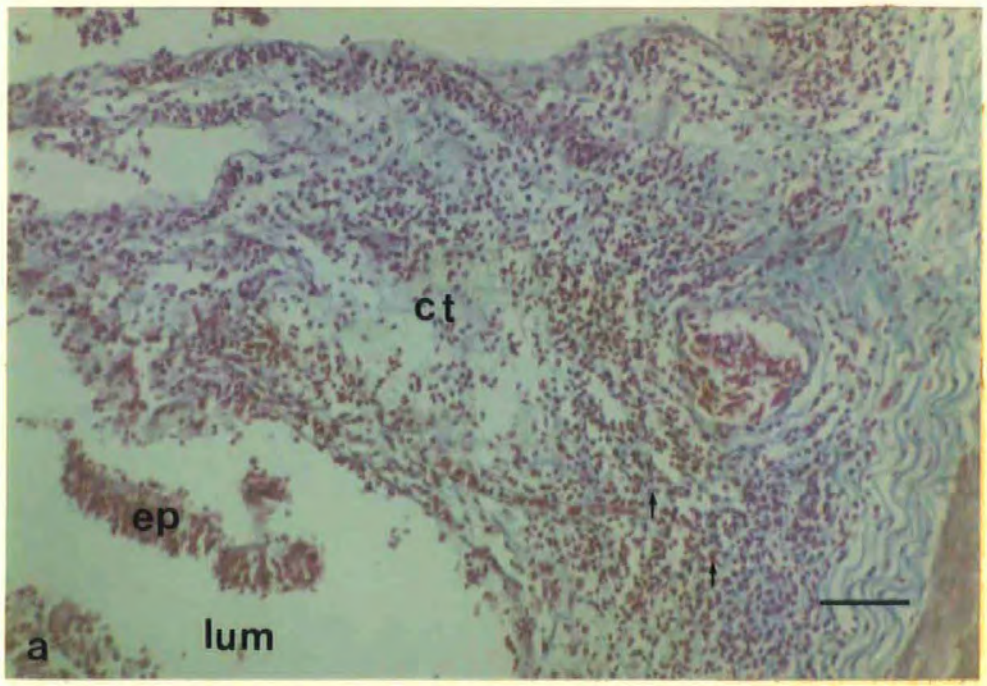

N
rer
bv
b

Plate 14 a) Light micrograph of infected eel gut (transverse section) showing cellular infiltration (arrowed) of intestinal connective tissue (ct) and epithelial detachment into the lumen (lum) (Mallory's triple stain, x 238, bar=50μm).

Plate 14 b) Light micrograph of infected eel oesophagus (transverse section) demonstrating fragmentation of epithelia (ep) and cellular infiltration (arrowed) of connective tissue (ct) (Mallory's triple stain, x 94, bar=100μm).

cm=circular muscle

lum=oesophageal lumen.


ct
ep
lum
a

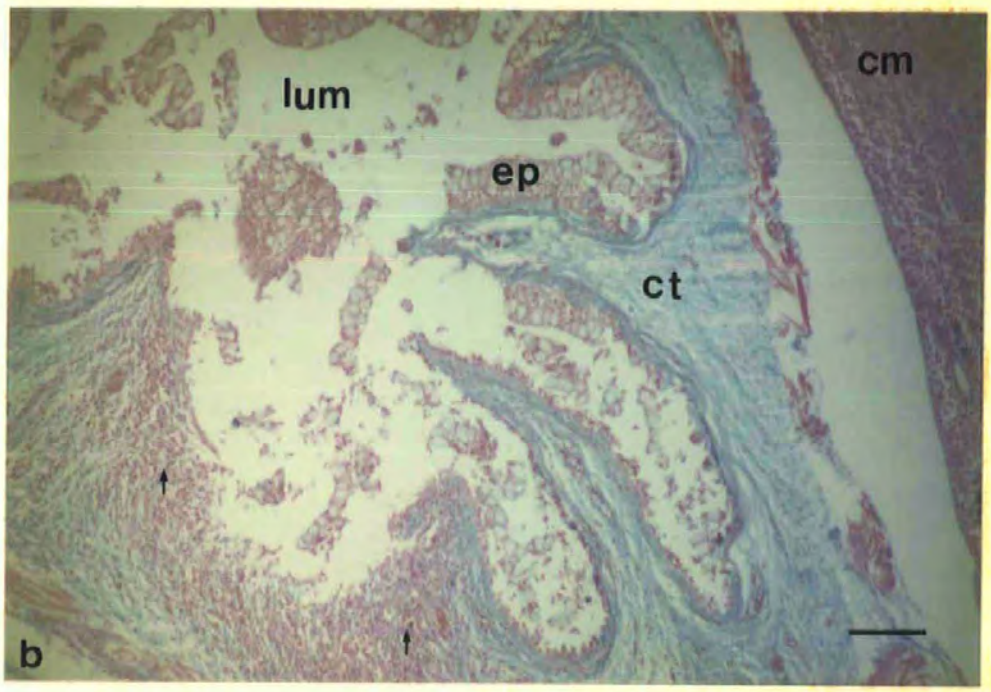

lum
ep
cm
ct
b

Plate 15 a) Light micrograph of infected eel gastric caecum (transverse section) showing fragmentation of detached epithelia(arrowed)(Mallory's triple stain,x 94,bar=100μm).

cm=circular muscle

ct= connective tissue.

Plate 15 b) Light micrograph of control gastric caecum (transverse section) showing normal epithelia(arrowed) (Mallory's triple stain, x 94,bar=100μm).

cm=circular muscle

ct=connective tissue.

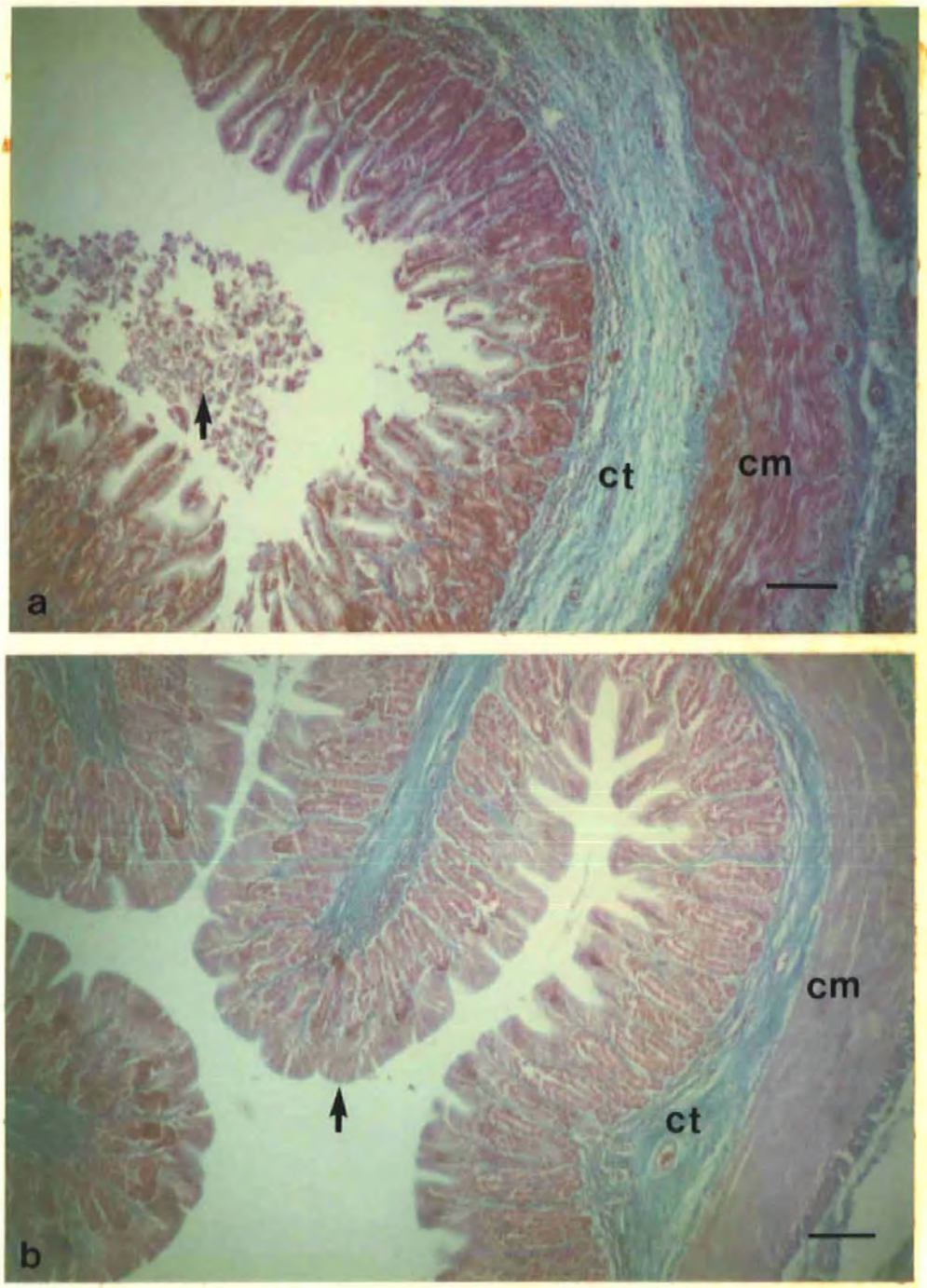
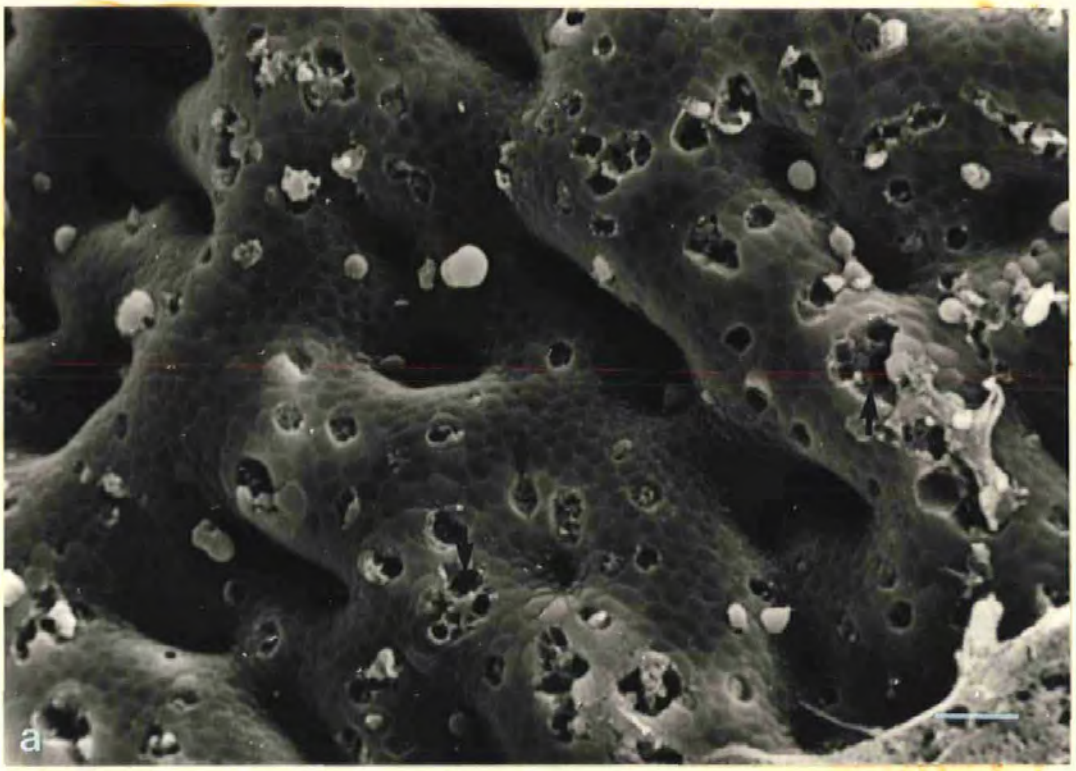

ct
cm
a
cm
ct
b

Plate 16 a) Scanning electron micrograph of infected eel gastric caecum showing necrotic pits(arrowed) in the gastric mucosa(x 1110,bar=10μm).

Plate 16 b) Scanning electron micrograph of control eel gastric caecum(x 400,bar=50μm).


a

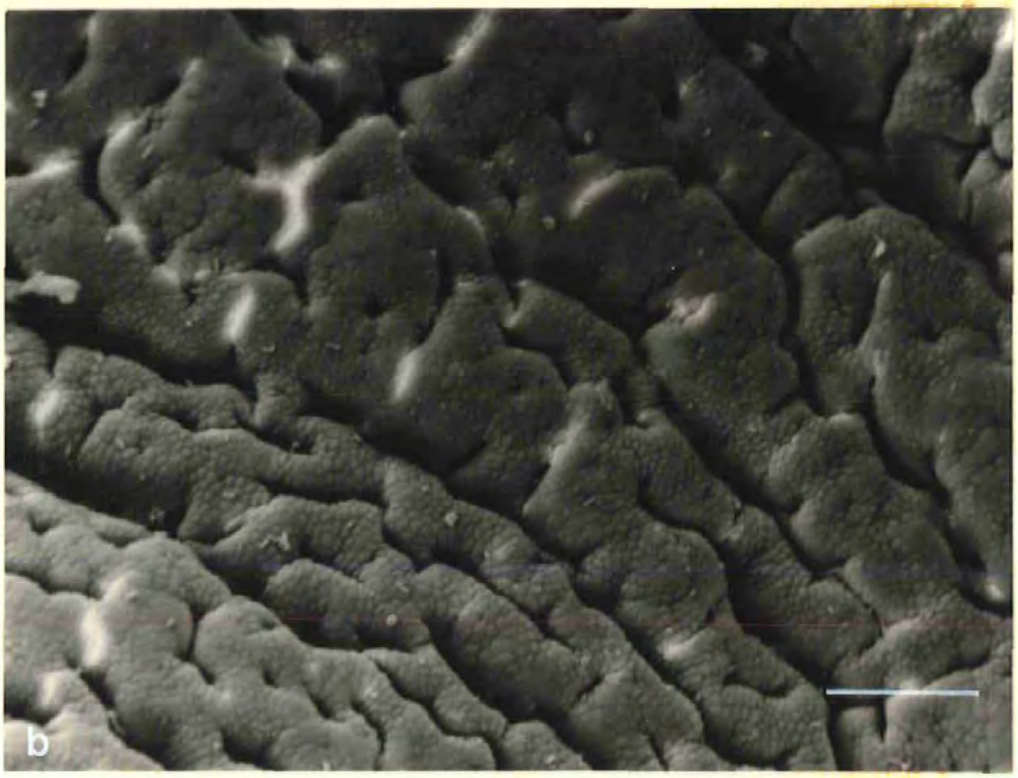

b

Plate 17 a) Light micrograph of infected eel gut (transverse section) demonstrating epithelial detachment (ep) from connective tissue (Mallory's triple stain, x 94, bar=100μm).

Plate 17 b) Light micrograph of control eel gut (transverse section) (Mallory's triple stain, x 94, bar=100μm).

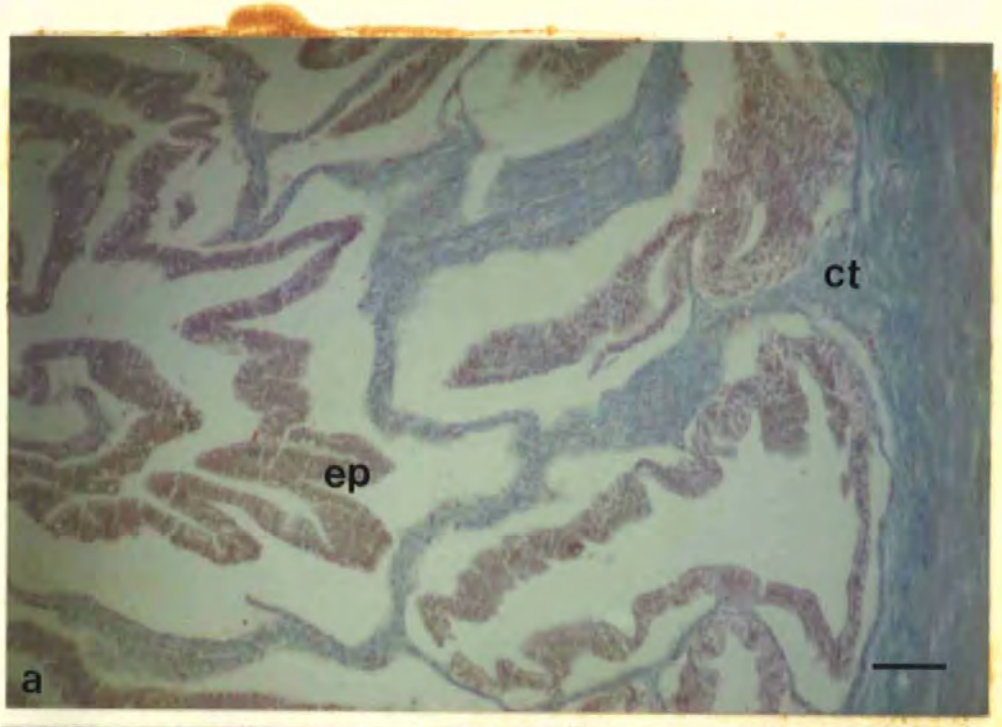
ep=epithelium

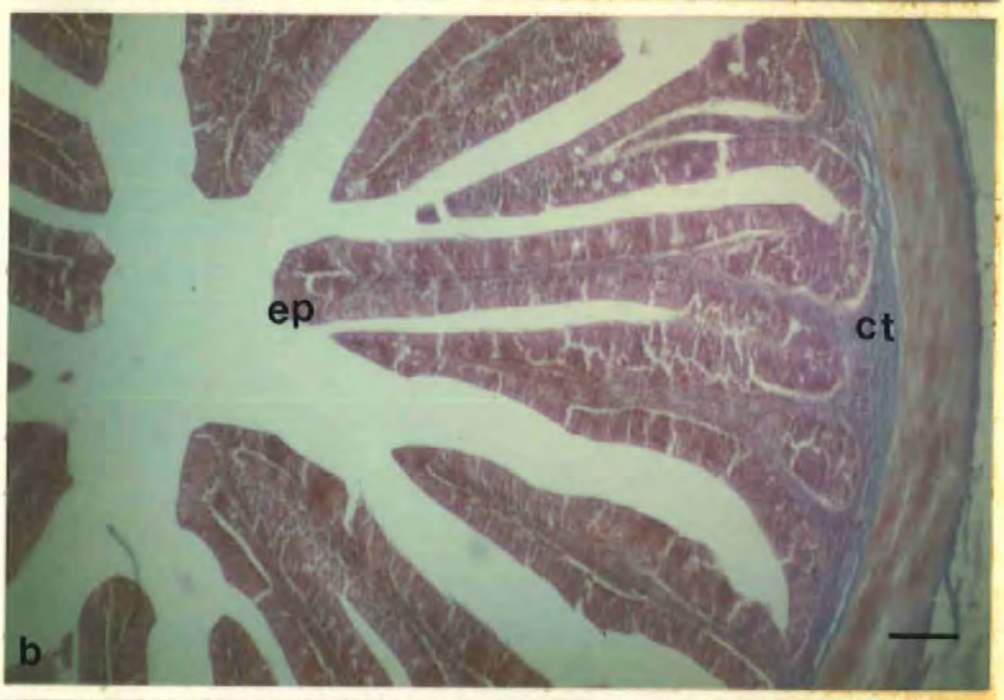
ct=connective tissue.

Plate 17 c) Electron micrograph of control eel gut indicating basement membrane (arrowed) (x 4160, bar=2μm).

m=mucosa

mv=microvilli.


ct
ep
a


ep
ct
b

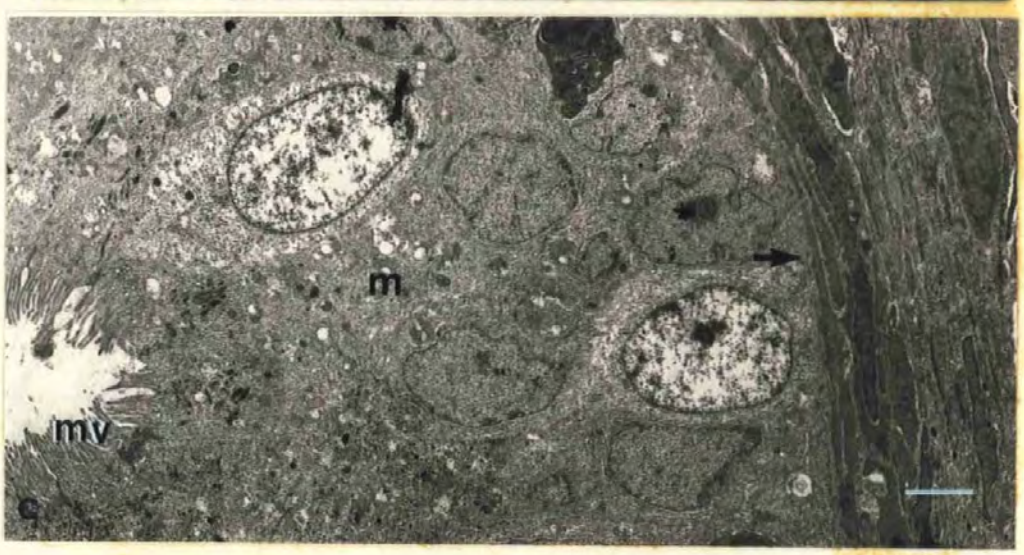

m
mv
c

Plate 18 a) Light micrograph of infected eel spinal column (transverse section) demonstrating haemorrhaging of the spinal cord (arrowed) (Mallory's triple stain, x 238, bar=50μm).

Plate 18 b) Light micrograph of control eel spinal column (transverse section) (Mallory's triple stain, x 238, bar=50μm).

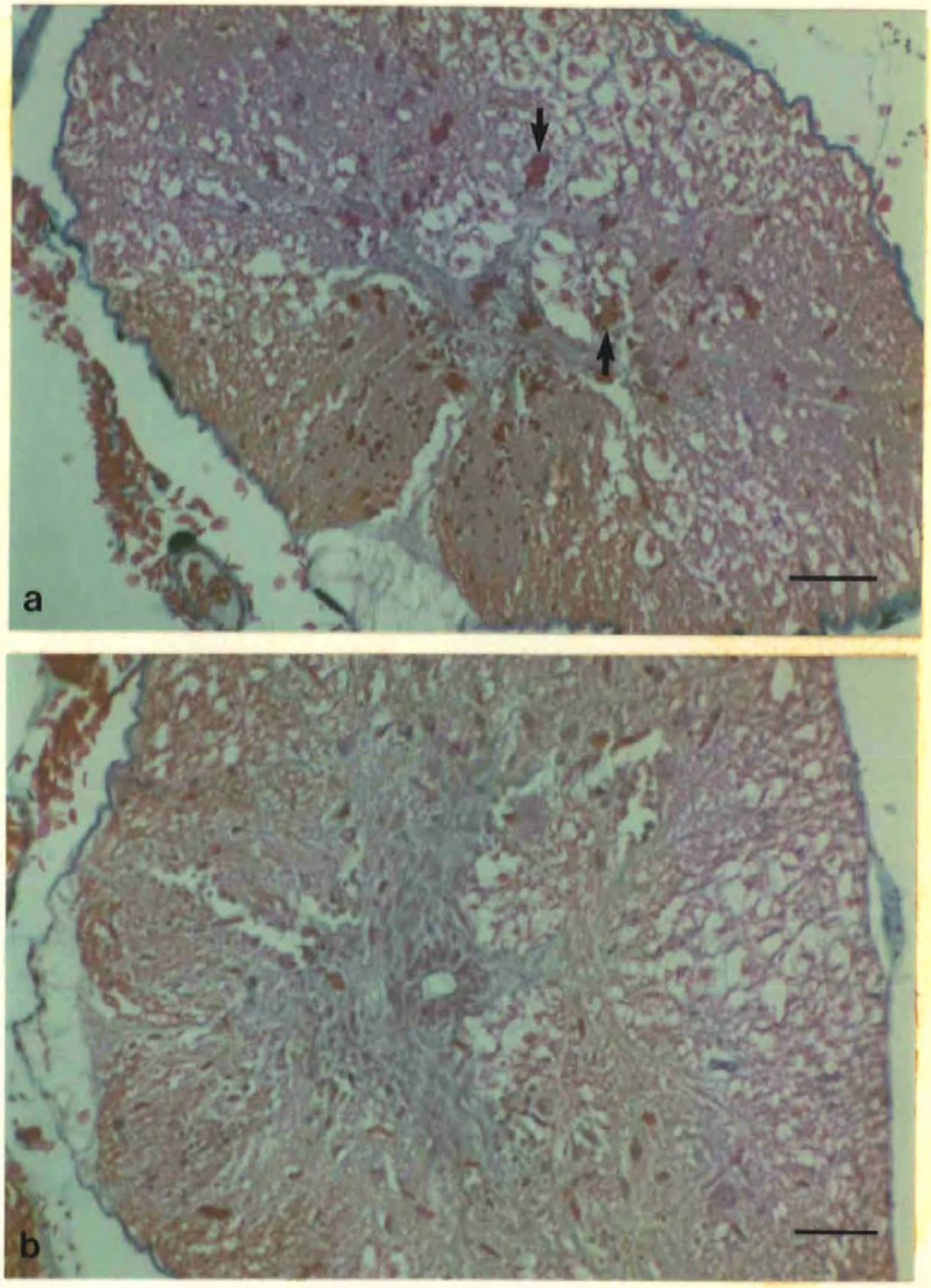

a
b

Plate 19a) Electron micrograph of bacteria(arrowed) between cardiac muscle fibres(cm) (x 11929,bar=1μm).

Plate 19b) Electron micrograph of infected eel gill demonstrating systemic bacteria(arrowed) invading adjacent tissues (x 2965,bar=3μm).

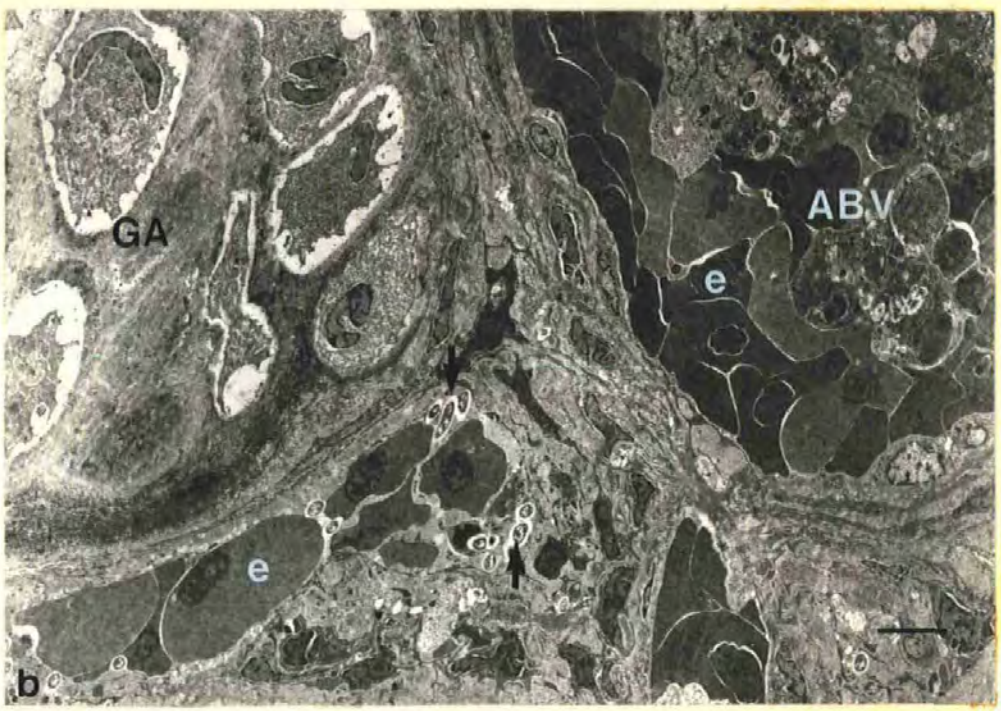
ABV = afferent blood vessel

GA = gill arch

e = erythrocyte.

Plate 19c) Electron micrograph of infected eel ventral fin demonstrating bacteria(arrowed) (x 19530,bar=0.5μm).


a
m
cm


GA
ABV
e
e
b

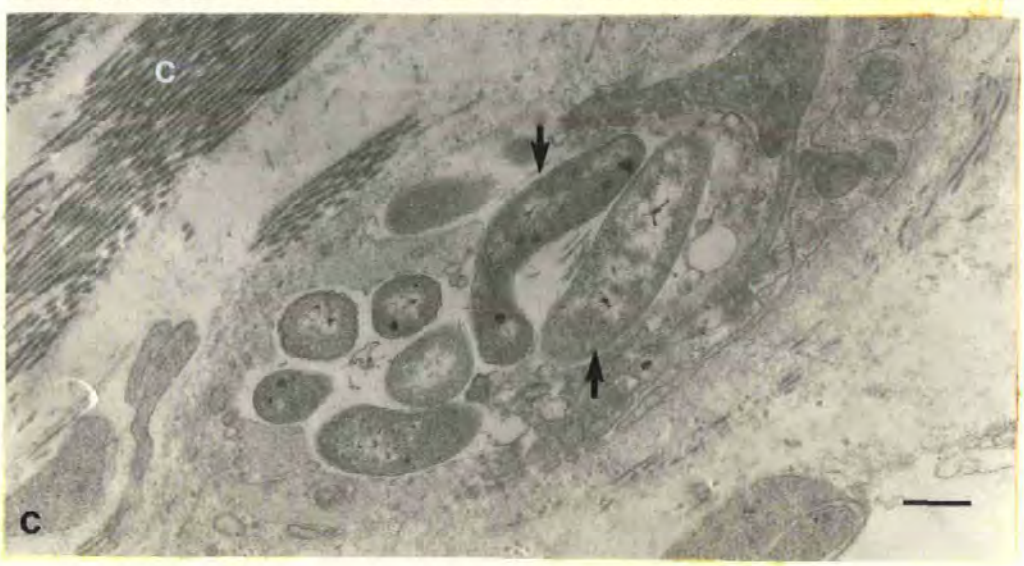

c
c

Plate 20 a) Electron micrograph of anal exudate demonstrating gut epithelium(x 8910,bar=1μm).

mc=mucous cell

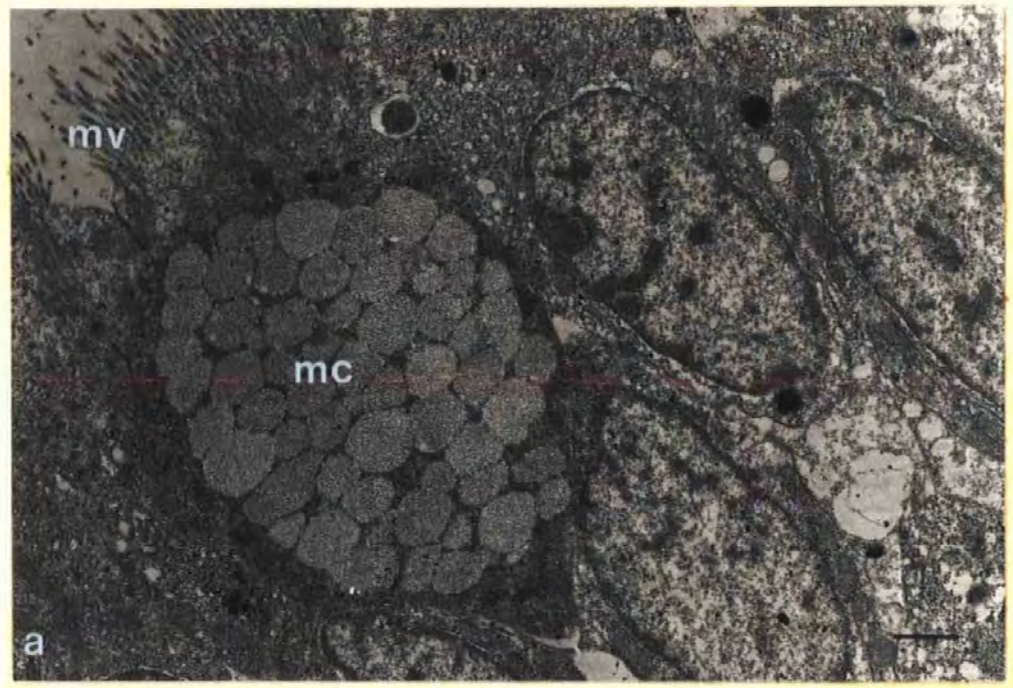
mv=microvilli

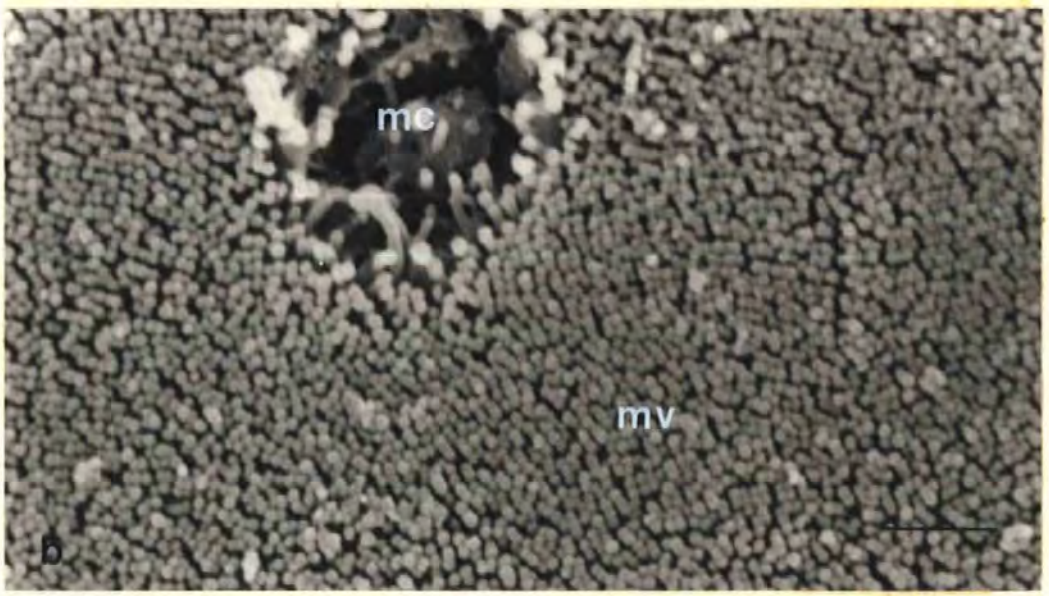
Plate 20 b) Scanning electron micrograph of control eel mucosa from lower gut region(x 16000,bar=1μm).

mc=mucous cell

mv=microvilli.

Plate 20 c) Light micrograph of resin section of anal exudate showing 'sheets' of epithelia(e)(x 792,bar=10μm).


mv
mc
a


mc
mv
b


e
c

Plate 21 a) Edible frog experimentally infected with vibriosis showing haemorrhaging of heart(h) and liver(l)(Mag x 1).

Plate 21 b) Light micrograph of infected frog gut(transverse section) demonstrating cellular infiltration (arrowed) of connective tissue(ct)(Mallory's triple stain, x 94,bar=100µm).
ct=connective tissue
e=epithelium.


h
l
a

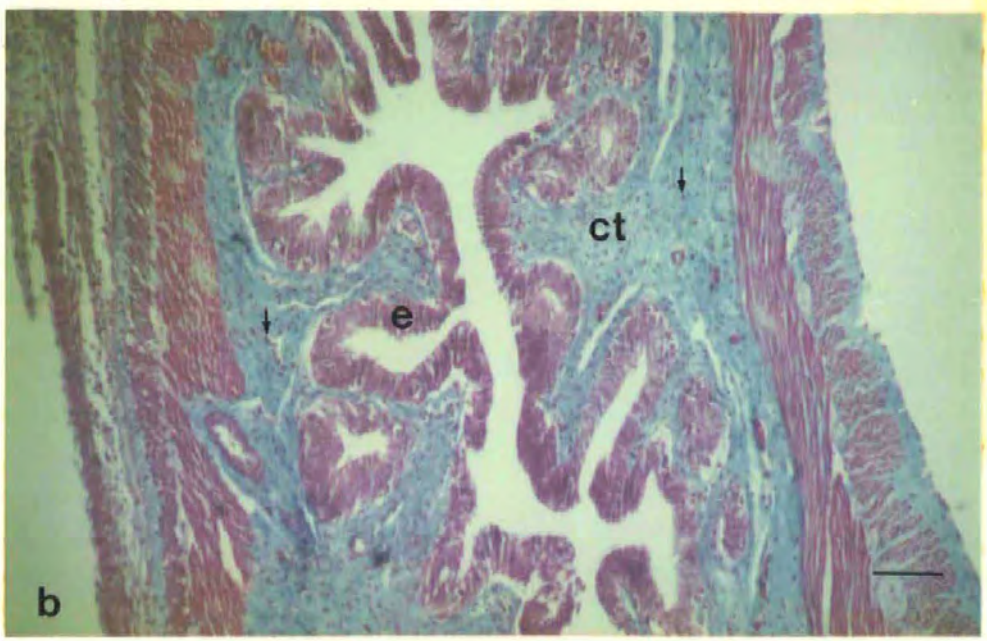

ct
e
b

Plate 22 Light micrograph of eel kidney demonstrating retention of colloidal carbon particles(arrowed)(Mallory's triple stain, x 94,bar=100μm).

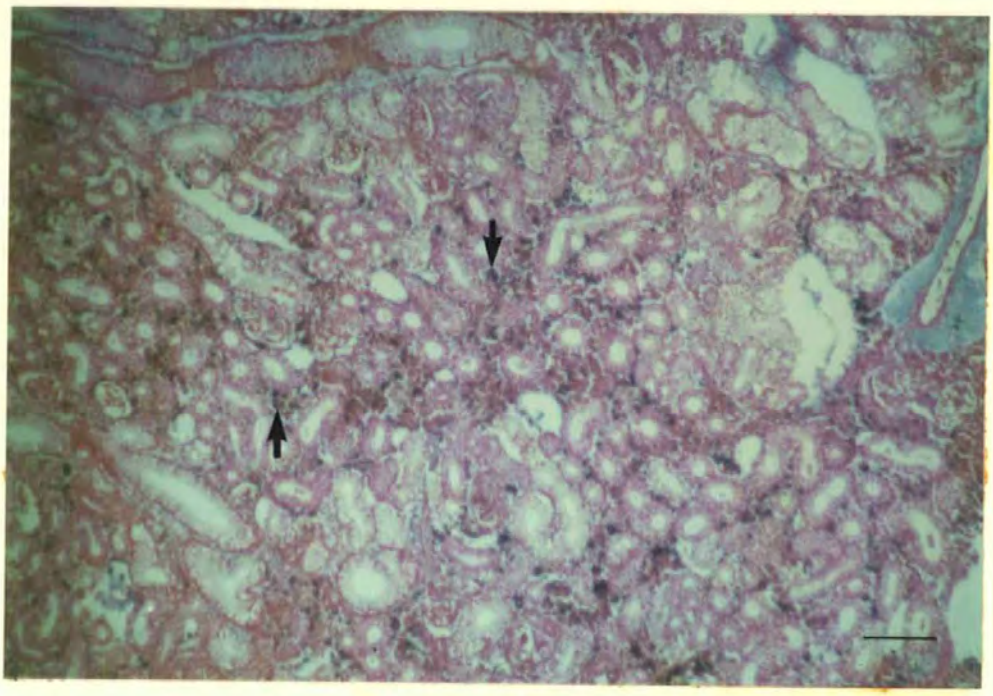


Plate 23 a) Light micrograph of infected liver demonstrating haemosiderin deposits(blue areas arrowed)(Perls' prussian blue x 238,bar=50μm).

Plate 23 b) Light micrograph of control spleen showing haemosiderin deposits(blue areas arrowed) in melanomacrophage centres(Perls' prussian blue, x 238,bar=50μm).

Plate 23 c) Light micrograph of control kidney demonstrating haemosiderin deposits(blue areas arrowed) in melanomacrophage centres(Perls' prussian blue, x 94,bar= 100μm).

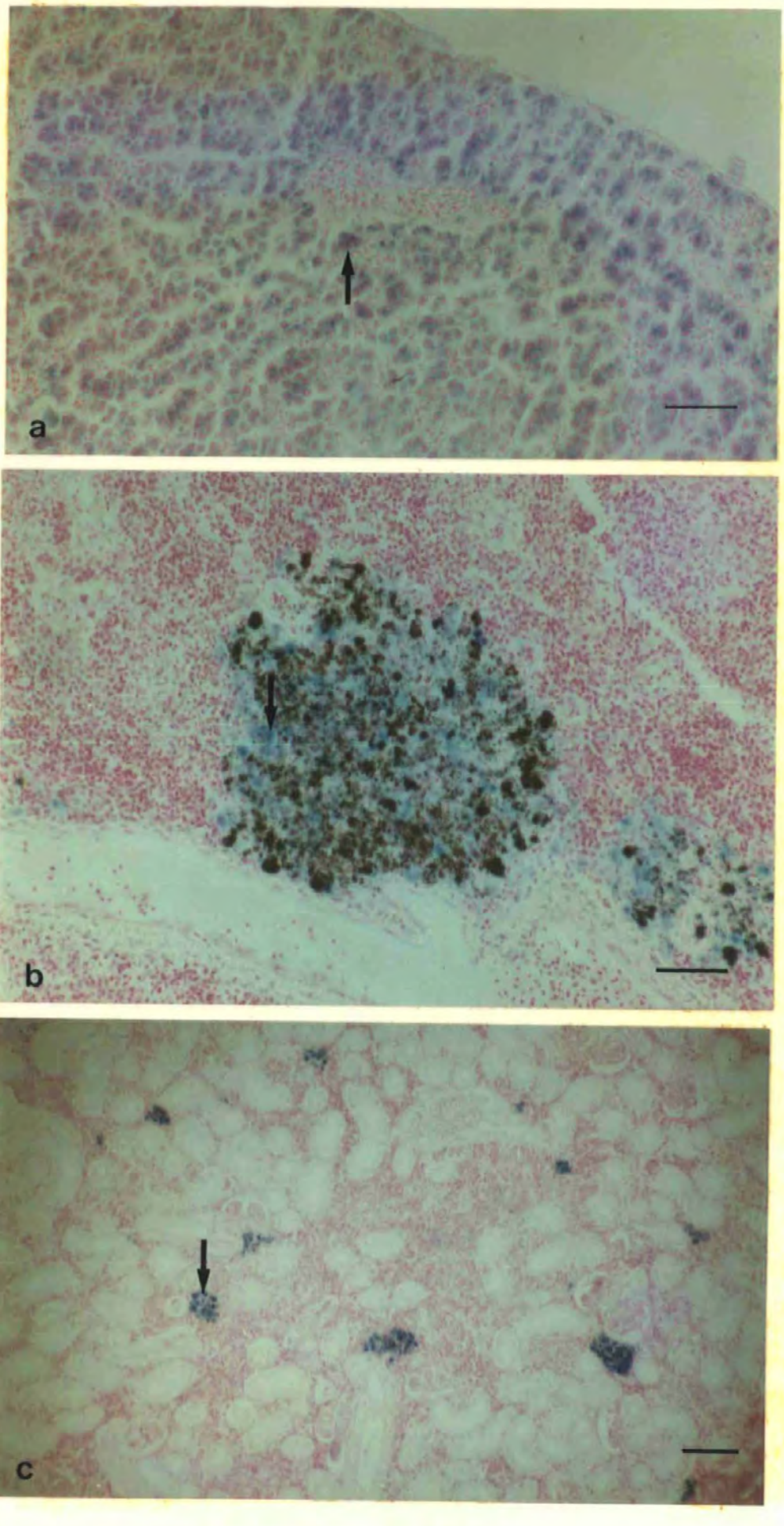

a
b
c

Plate 24 a) Protein profile of eel plasma revealing apparent reduction of band coinciding with human haptoglobulin(arrowed) in infected samples.

Tracks 1-4 = Infected plsma

Tracks 5-8 = Control plasma

Track 9 = Human plasma

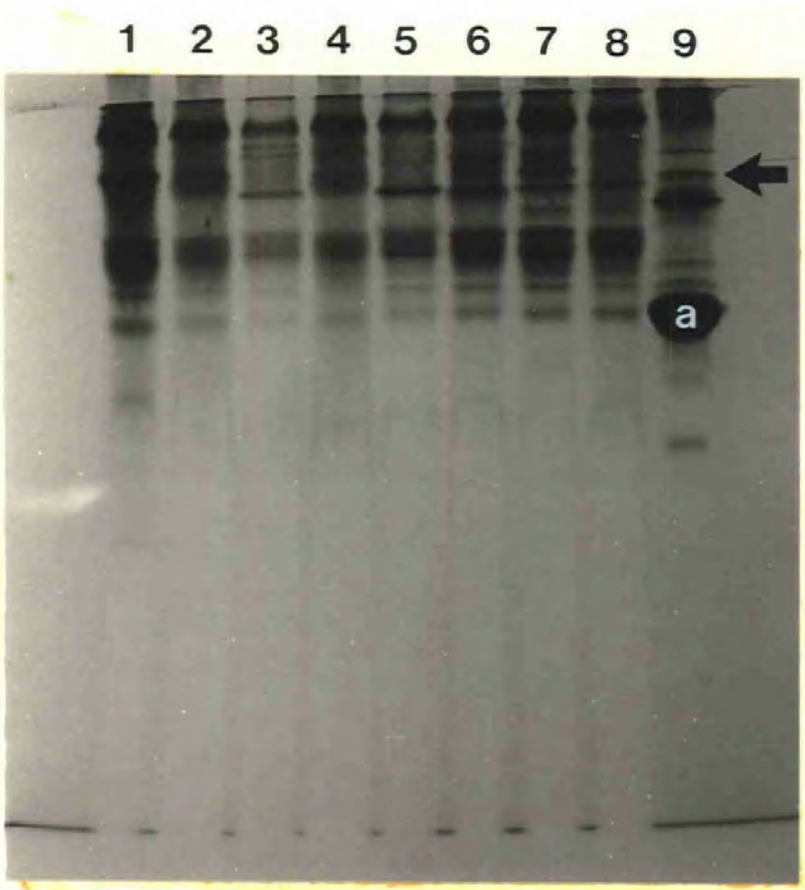
a = Albumen.

Plate 24 b) Protein profile of eel plasma separated in the presence of 3% SDS showing absence of polypeptides of 47000 and 43000 daltons(⇨) and 36500 daltons(⬅),and an additional polypeptide of 39000 daltons(←) in infected samples.

Tracks 1-4 = Infected plasma

Tracks 5-8 = Control plasma

Track 9 = Protein standards: I=phosphorylase b (94000),II=albumen(67000),III=ovalbumen(43000), carbonic anhydrase(30000),IV=trypsin inhibitor (20100),V=lactalbumen(14400).


1 2 3 4 5 6 7 8 9
a

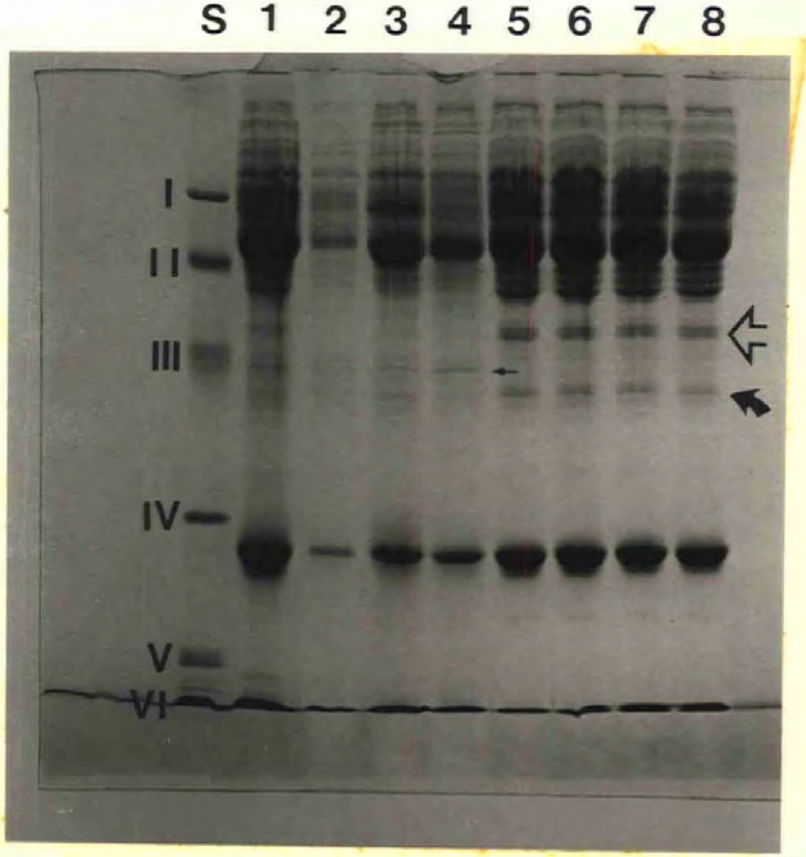
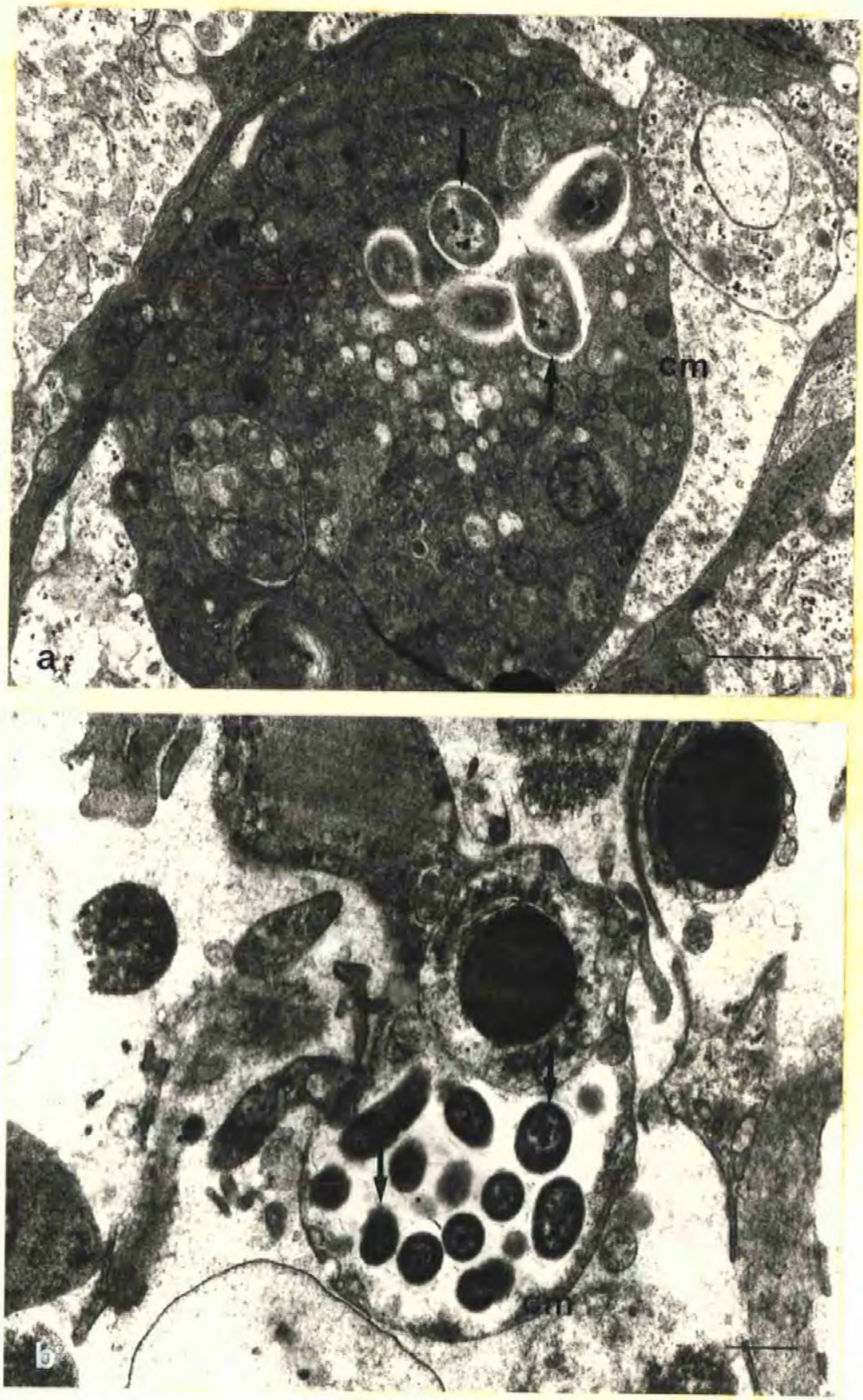

S 1 2 3 4 5 6 7 8
I
II
III
IV
V
VI

Plate 25 a) Electron micrograph of intracellular bacteria(arrowed) in liver cell(x 16500,bar=1μm). cm=cell membrane.

Plate 25 b) Electron micrograph of intracellular bacteria(arrowed) in spleen cell(x 11700,bar=1μm). cm= cell membrane.


cm
a
cm
b

4.1.0 INTRODUCTION

In order for an organism to be pathogenic it has to be capable of infecting a susceptible host. Bacteria cause disease by either invasion of the tissues and/or body surfaces which lead to damage of host cells in the immediate vicinity; or production of potent toxins which may act at remote sites (Davis et al., 1980). Most infections start on the mucous membranes of the respiratory, alimentary and urinogenital tracts (Smith, 1978). Because of the nature of their environment, aquatic animals have in addition, direct contact with large numbers of water-borne, potentially pathogenic, organisms at all body surfaces. A prerequisite for infection of host body surfaces is attachment in the face of commensal bacterial competition, physical dislodgement processes and adverse environmental conditions. For example infection via the intestinal tract in mammals is affected by the presence of mucus, acid, enzymes and bile. Mucus protects epithelial cells and contains secretory immunoglobulin which protect the host from infection. Microorganisms infecting by the intestinal route are often capable of surviving in the presence of acid, proteolytic enzymes and bile (Mims, 1977). Normal gut commensal bacteria (Streptococcus faecalis, E. coli, Proteus and Pseudomonas spp) as well as gut pathogens (Salmonella and Shigella spp) grow in the presence of bile (Mims, 1977).

The present chapter considers possible routes by which V. anguillarum may enter the fish host (2.10.2, 2.10.3 and 2.10.7); and how this organism may be suited to exist in the alimentary canal of fish as an opportunist pathogen (2.10.4 - 2.10.6 and 2.12.8).

As a consequence of maintaining wild eels in captivity, this species ceased to feed. Although the main experimental animal has

been the eel, other species have been employed for studies concerning feeding aspects of disease.

In connection with work on host responses to infection (2.4.1), it was found that the presence of V. anguillarum in the eel tissues initiated a rapid deposition of haemosiderin (2.7.2) in liver parenchymal cells; this reaction was used as a means of detecting bacterial presence in fish tissues and to supplement bacteriology (2.2.6).

4.2.0 RESULTS

4.2.1 External challenge with V. anguillarum

Eels challenged by strains (UNH 569 and PB-15) in the surrounding medium (2.10.1) did not succumb to vibriosis. After three weeks, V. anguillarum was shown to be present in blood, kidney and hind gut of these fish, with evidence of entry of host tissues supported by demonstration of haemosiderin deposits in liver sections (2.7.2).

4.2.2 Feeding fish with contaminated diet

Blennies, mullet and rockling fed diet containing V. anguillarum (2.10.2) did not succumb to vibriosis within the duration of the experimentation.

4.2.3 Effect of inoculation route on vibriosis in eels

Fish injected, regardless of route (im, ip, iv, sc), died within two days of being inoculated (2.10.3), displaying typical manifestations of vibriosis and severe necrotic ulceration of skin and underlying musculature at the injection site. Eels receiving oral inocula or exposed to bacteria in the water (2.10.3), did not succumb to disease within three weeks of challenge. However, eels receiving

hind gut inocula died 15-16 days post-inoculation.

4.2.4 Entry of V. anguillarum as a consequence of osmoregulation

Dissection of fish after one day's exposure to water containing Aquadag (2.10.4) revealed that at salinities greater than $13^{o}/oo$ (40% seawater) suspended matter entered the fish gut. This was demonstrated in both grey mullet and eels. Eels subjected to similar salinity gradients, containing viable V. anguillarum (10^{6} cm^{-3}) were found on enumeration of gut bacteria (2.2.6) to contain V. anguillarum in all cases with largest numbers being encountered at 6.8 and $10.2^{o}/oo$ salinities (Table 7).

4.2.5 Gut commensal aspects of V. anguillarum

Strains cultured in TSB with a range of pH, (2.12.8) were found to have a mean pH optimum of 8.2 $\pm$ 0.4, with active growth taking place between pH 5.5 and 10.5 (Fig. 13). Growth in the presence of fish bile (2.12.8) revealed all strains tested to be resistant to eel, carp and plaice bile.

4.2.6 The ability of V. anguillarum to agglutinate erythrocytes

The ability strains to agglutinate guinea-pig, rabbit and eel erythrocytes was determined (2.12.7) with the result that 7 out of 10 strains agglutinated mammalian erythrocytes, while none of the strains agglutinated eel erythrocytes.

4.2.7 Fate of intestinal V. anguillarum

Following inoculation of the hindgut of eels with V. anguillarum and subsequent examination of these tissues by electron microscopy (2.10.5), it was revealed that high proportions of the inocula had

Table 7 Enumeration of bacteria in gut of eels exposed to salinity gradient containing V.anguillarum($10\ cm^{-3}$).

Salinity °/oo	Colony forming units gm^{-1}
0	1.52×10^{2}
3.40	5.64×10^{4}
6.80	2.87×10^{8}
10.20	1.06×10^{6}
13.60	3.86×10^{4}
17.00	3.87×10^{3}
20.40	8.67×10^{3}
23.80	6.92×10^{3}
27.20	4.67×10^{3}
30.60	8.51×10^{2}
34.00	2.39×10^{4}

Table 8 Occurrence of bacteria in tissues from coastal fish.

Species	Kidney	Gut	Liver	Spleen	Bile
Merluccius merluccius	-	+	+	+	-
Merlangius merlangus	-	+	+	-	+
Micromesistius poutassou	+	+	+	+	-
Molva molva	+	+	+	+	+
Scophthalmus maximus	+	+	+	+	-
Limanda limanda	+	+	+	-	-
Pleuronectes platessa	+	+	+	-	+
Platichthys flesus	+	+	+	-	+

left the hind gut within 2 days. Observations of hind gut segments by SEM demonstrated a small number of 'vibrio-like' bacteria (Plate 26b) which were present in similar numbers in segments taken 7 and 14 days post-inoculation. Examination of hind gut tissues from eels killed 2 days post-inoculation failed to reveal bacteria traversing the gut mucosa. The general appearance of inoculated gut mucosa closely resembled uninfected control tissues.

4.2.8 Status of *V. anguillarum* in wild fish

From a range of coastal fish examined (2.10.6) almost a thousand isolates were obtained. A small proportion were Gram negative, motile rods haemolytic on blood agar (2.2.4), but these isolates were found not to be sensitive to the vibriostatic compound O/129, and hence no members of the genus *Vibrio* were isolated from the fish examined. From these studies it was apparent that bacteria were present in the tissues as well as the gut of 'healthy' fish (Table 8).

4.2.9 Penetration of eel skin by water-borne bacteria

The ability of water-borne bacteria to penetrate excised eel skin was determined for strains of *V. anguillarum* (UNH 569, PB-15), and compared to the Gram negative, motile, marine bacterium : *Pseudomonas aeruginosa* (2.10.7).

Bacteriology revealed that *V. anguillarum* traversed the eel dermis, and electron microscopy demonstrated the presence of vibrio-like bacteria between collagen fibres (Plate 26a). Both strains were found to traverse the skin, but *P. aeruginosa* was shown to be unable to do so. Comparisons made between control and experimental skin preparations by electron microscopy revealed no major degeneration of tissues during experimentation.

4.3.0 DISCUSSION

Initial challenge experiments with *V. anguillarum* demonstrated a dramatic difference between eels and mullet in their susceptibility to vibriosis. The reasons for the death of mullet following short exposure to *V. anguillarum* compared with the tolerance of eels to bacterial presence, are unclear; however these fish species are very different anatomically and ecologically. Inter-strain differences in host specificity have been suggested previously (Antipa, 1976; Egidius & Anderson, 1977).

Although eels did not succumb to vibriosis when challenged by water-borne organisms, it was evident that bacteria entered the tissues with subsequent accumulation in the kidney. Death of eels receiving injected inocula and those surviving following challenge by water-borne bacteria may be related to the number of bacteria involved. Small numbers of tissue bacteria appear to be tolerated while large numbers lead to disease.

Failure to produce disease in fish receiving oral inocula and fed contaminated feed-stuffs was surprising, as Rucker (1959) and Ross *et al.* (1968) implicated contaminated diets as responsible for subsequent outbreaks of vibriosis; while ingestion of *A. salmonicida* was thought to be the main route of entry of bacteria in fish dying of furunculosis (Klontz *et al.*, 1966).

It would have been of interest to repeat the experiments concerned with feeding injected mullet to higher carnivores, but using fish which had been infected by water-borne bacteria as opposed to being injected with broth cultured *V. anguillarum*. The bacteria in naturally infected mullet may be more virulent as a consequence of selection pressures during infection.

In addition to bacteria entering fish through feeding, the

present experiments suggested that bacteria could enter the fish gut due to osmoregulatory functions. As fish tissues are hypo-osmotic to the marine environment they have to regulate their osmotic balance by continual drinking of the medium and excreting the inherent salts. Both eels and mullet were found to imbibe Aquadag placed in the tank water when the salinity was raised above 13.5°/oo (40% sea water). Substituting the dye with viable bacteria gave confusing results, in that bacteria entered the gut at all salinities but with a generally higher intake between 3.4°/oo and 10.2°/oo salinities. Why the number of bacteria showed no correlation with the passive dye experiments is unclear but would tend to suggest that entry by bacteria into the fish was not directly related to osmoregulation. Experiments carried out by Keys (1933) showed that eels did drink the medium when placed in full strength sea water; blocking of the oesophagus led to gradual weight loss and eventual death. Shaw (1960) further showed that, in sea water eels drank up to 200 cm^3 water kg^{-1} day^{-1}. By these criteria the eels in the highest salinity in the present studies would consume approximately 14 cm^3 of the medium which is equivalent to approximately 1.4×10^7 viable bacteria, or about 10^6 gm^{-1} of gut. A possible reason for the lower numbers of bacteria in the higher salinities could be due to the permeability of the gut mucosa. Hirano and Utida (1971) and Hirano and Mayer-Gostan (1976) showed that at higher salinities the eel gut became more permeable than at low salinities. Thus at the higher salinities gut bacteria may possibly pass through the gut epithelium and into the tissues more rapidly.

Inoculation of the hind gut of eels with V. anguillarum was the only route by which disease could be produced other than by infection by injection of the tissues. Bacteria inoculated via the cloaca took

longer to cause disease than injected inocula. However this would be expected since infection by this route would require time for invasion mechanisms to develop. SEM observations of the gut following inoculation of the hind gut with V. anguillarum showed a small number of vibrio-like bacteria on the gut mucosa after two days, and that most of the inoculum had left this location. Although bacteria were not observed in gut sections, mucosal traversal may have taken place. Experiments examining the response of the frog (Rana temporaria) gut to inoculated V. cholerae were carried out by Avtsyn et al. (1979) with the result that this organism was shown to readily traverse the gut wall and enter the tissues, with V. cholerae being isolated from liver and bile. Gut traversal by V. cholerae in frogs thereby demonstrated that a close relative of V. anguillarum was capable of invading a poikilothermic animal across the mucosa which seems a feasible route of entry for V. anguillarum.

Tolerance of V. anguillarum to bile and a wide range of pH conditions would suggest that this bacterium is particularly well suited to a gut existence and could survive in the fish gut. It has been shown that V. cholerae has the ability to remain viable in the gall bladder of humans and monkeys (Benenson, 1970) and V. parahaemolyticus can grow in the presence of bile (Burrows & Miller, 1976), suggesting that bile tolerance is a common feature of this genus. A large proportion of strains tested were found to cause haemagglutination, a feature indicative of mucosal adherence in V. cholerae (Bales & Langford, 1961); further supporting the theory that V. anguillarum may be gut-associated. The influence of pH and bile have been suggested to be major selection forces in bacterial populations entering fish from the external medium (Sera & Kimata, 1972; Sera & Ishida, 1972a,b & c; Sera et al., 1972).

Experiments involving the penetration of eel skin *in vitro* demonstrated that *V. anguillarum* was capable of penetrating the dermis, a phenomenon not exhibited by *Ps. aeruginosa*. Although this indicates a possible route of entry for *V. anguillarum* to eel tissues, it must be emphasised that excised eel skin removed from the animal behaves differently as compared with the living tissue. In healthy eels mucus, produced continuously, provides a flushing mechanism preventing bacteria settling on the skin. Under stress conditions, mucus production becomes retarded, at which times opportunist bacteria could gain access to the tissues. In addition, when the skin becomes physically damaged by abrasions, ecto-parasites such as *Lepeophtherius salmonis* (Håstein & Bergsjo, 1976) and *Argulus* spp (Griffin, 1953) or by tagging lesions (Roberts *et al.*, 1973) bacteria could traverse this barrier. Penetration of the fish dermis as a route of infection by *Flexibacter columnaris* was reported by Wood (1974).

Traversal of the gills as a means of entering fish tissues was not examined here because of the complexity of such studies, however, bacteria (Bowers & Alexander, 1981) and viruses (Ahne, 1978) have been shown to enter fish across the gill.

The presence of bacteria in apparently healthy fish has been reported elsewhere (Evelyn & McDermott, 1961; Bullock & Snieszko, 1969; Chung & Kou, 1973) and is endorsed by the results obtained from examination of coastal fish analysed here. Fish have been shown to have several mechanisms for removing foreign matter from the circulation, one of which involves renal excretion (Pritchard *et al.*, 1980) which could account for the presence of bacteria in the eel kidney. The present studies, supported by the literature, would strongly suggest that fish tissues are not free from bacteria as is generally the case in higher vertebrates, and that fish can tolerate the presence of

systemic and tissue bacteria (50 gm^{-1}, 6.2.3) which could be opportunist pathogens.

Bacteriological examination of the marine environment has shown that bacteria exist in aquatic animals at all trophic levels. Of the bacteria isolated, the genus Vibrio has been frequently encountered (Liston, 1957; Baross, 1973; Simidu et al., 1973; Trust & Sparrow, 1974) and has been found to be the dominant genus in red sea bream (Chrysophrys major) (Sera & Ishida, 1972a & b) grey mullet (Mugil cephalus) (Mowlan et al., 1979) and salmon (Yoshimizu et al., 1976a & b; Yoshimizu & Kimura, 1976). Vibrio spp have been isolated from plankton (Simidu et al., 1971), molluscs (Colwell & Liston, 1960; Beeson & Johnson, 1967; Lovelace et al., 1968), crustacea (Yashuda & Kitao, 1980) and several other invertebrates (Colwell & Liston, 1962; Håstein & Holt, 1972; Grischowsky, 1973). With fish of commercial importance tending to be top carnivores in the aquatic environment it would seem likely that Vibrio spp would enter fish during feeding.

Although most studies in the literature have not classified to the species level, there have been reports of V. anguillarum being carried by herring (Wood, 1974) and red sea bream (Hirano & Yone, 1971). Vibrio spp were not isolated in the present study suggesting that geographical location and climate may influence the aquatic flora. Although the work suggests that V. anguillarum could be a 'commensal', it has been shown that commensal bacterial speciation depends on the external environment of the fish (Venkataraman & Sreenivasan, 1953; Potter & Baker, 1961). Also the bacterial speciation may be influenced by the feeding status of the fish, as Margolis (1953) has shown that fasting fish lose their gut flora.

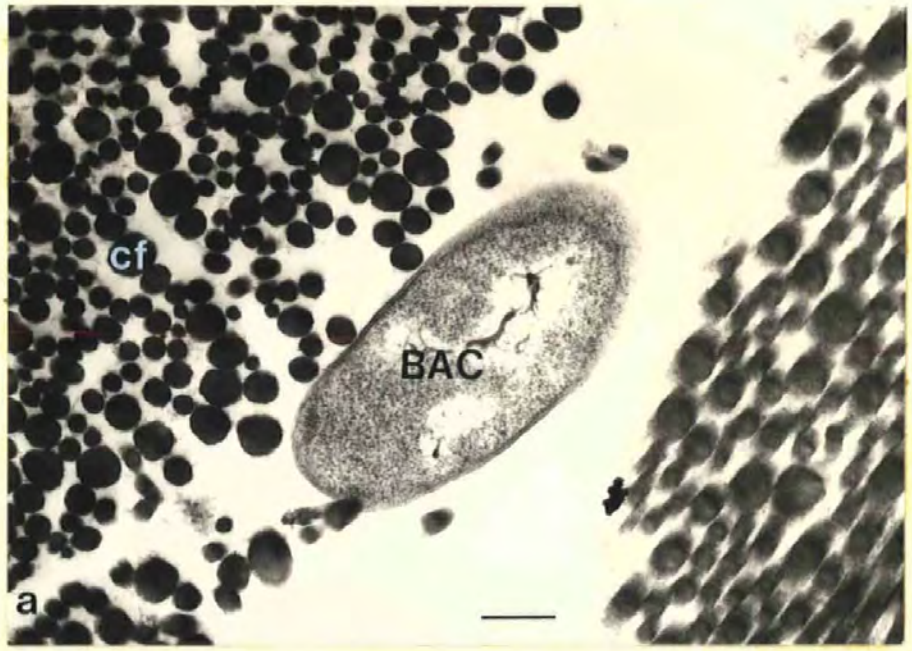
In summary it would seem that fish challenged by environmental

V. anguillarum became infected by this bacterium with disease being related to the ability of the host to tolerate bacterial presence. The eel is a particularly resilient species (Larsson & Fänge, 1969) and may be more resistant to infection by this organism than other fish.

Although these studies require further investigation, they would suggest that V. anguillarum could enter the digestive tract of fish and survive the extreme environment of the foregut, and that infection probably occurs following traversal of the hindgut mucosa. Latency studies were not fully investigated, but a latent infection would seem a possible cause of the sudden mass mortality experienced during epizootics of vibriosis.

Plate 26 a) Electron micrograph of excised eel skin demonstrating presence of 'vibrio-like' bacteria(BAC) between collagen fibres(cf)(x 50000,bar=0.2μm).

Plate 26 b) Scanning electron micrograph of eel hind-gut showing presence of 'vibrio-like' bacteria(BAC) on the gut mucosa(x 16666,bar=0.6μm).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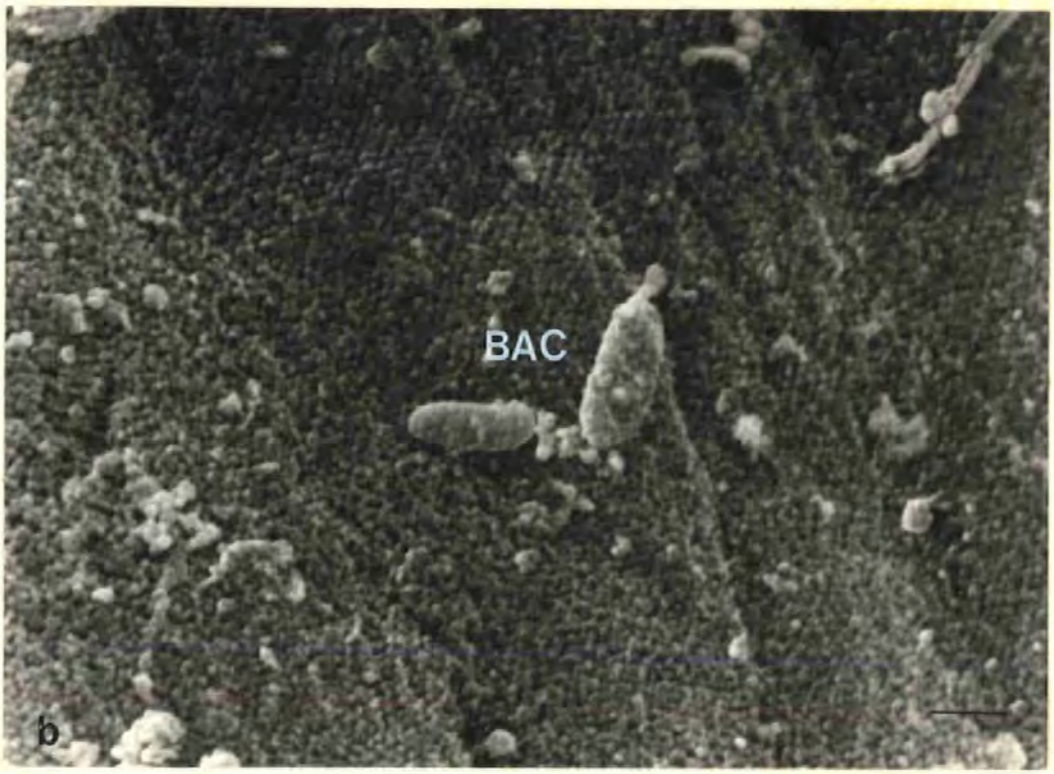
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5.1.0 INTRODUCTION

Disease production in fish is similar to the process in other animals in that it is more complicated than mere contact between host and pathogen, but is particularly dependent on environmental factors (Snieszko, 1958, 1964). Environmental pressure which requires physiological compensation is termed 'stress' (Hoar, 1966) which in fish has been shown to involve hormonal changes, particularly catecholamines and corticosteroids (Mazeaud et al., 1977), but has complex effects on the fish.

Under intensive culture conditions fish are predisposed to stress of overcrowding, handling and transportation. How environmentally induced stress relates to vibriosis appears to have been poorly studied, although temperature and handling has been implicated in increasing susceptibility to this disease (Ross, 1970; Fryer et al., 1972; Wood, 1974).

The aims of the work described in the present chapter were to elucidate the influence of environmental variables on the V. anguillarum-eel interaction and to observe the effect of host weight on pathogenesis. Discussions on stress have been made with reference in particular, to the work of Mazeaud et al. (1977) which has been diagrammatically represented in Fig. 8.

Throughout the work reported in this chapter V. anguillarum strain UNH 569 was used unless otherwise stated.

5.2.0 RESULTS

5.2.1 Effect of environmental temperature on pathogenesis

Eels acclimated to a range of ambient temperatures were observed

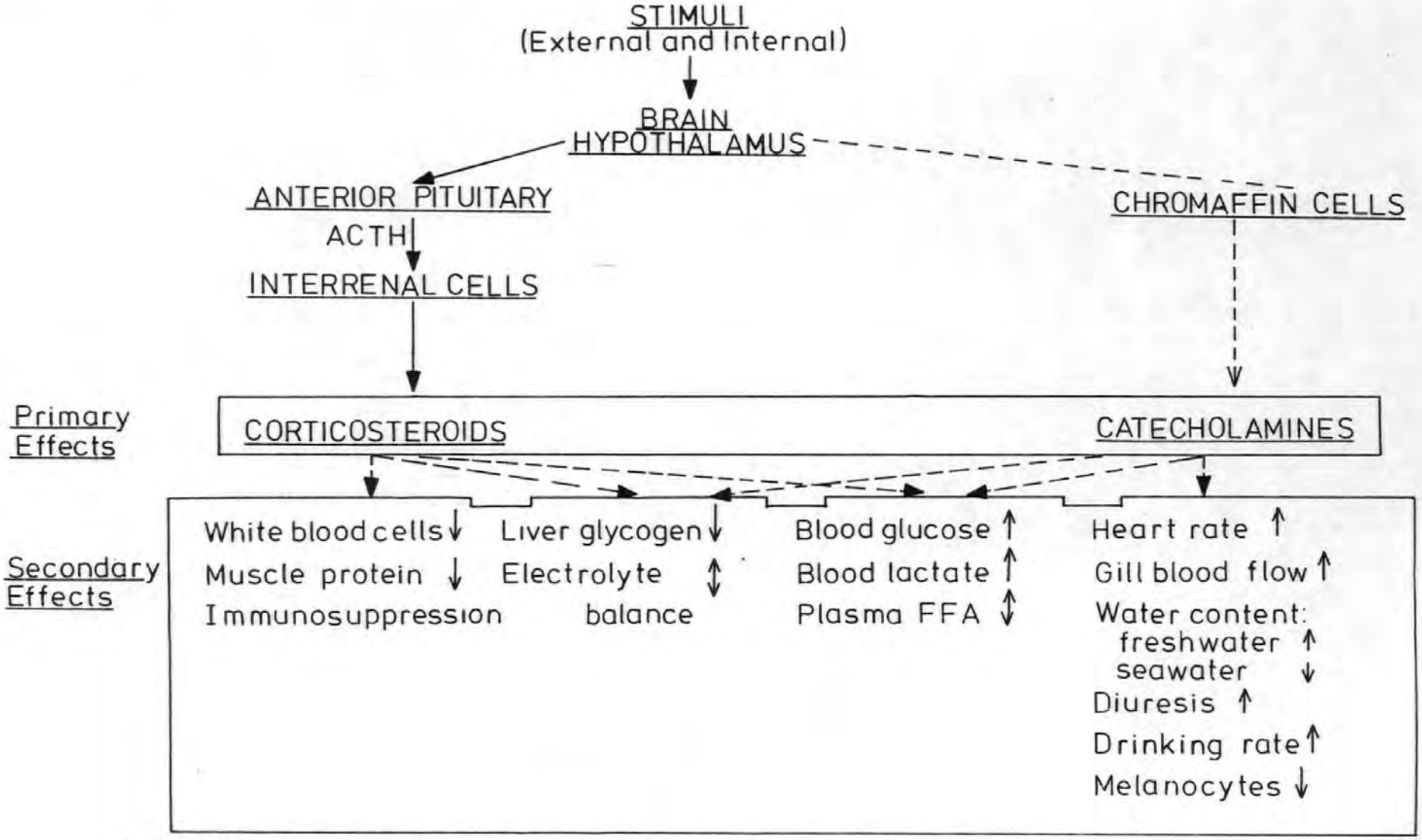


Fig 8 Scheme incorporating current information on the interelationship between primary and secondary effects of stress in fish.

after being inoculated with V. anguillarum (2.11.1); of the eels maintained at 37°C, three died prior to being inoculated. None of the fish kept at 5°C became diseased in 30 days. Eels maintained at 10°C died approximately three weeks post-inoculation, while fish kept at 20°C and above died up to 8 days post-inoculation. Mortality data were displayed graphically (Fig. 9) showing a correlation between death time and temperature following experimental infection. Apart from the eels at 37°C dying prior to injection with V. anguillarum, identification tests (2.2.4) confirmed death by vibriosis.

5.2.2 Effect of water salinity on vibriosis

Infecting eels acclimated to a salinity gradient (2.11.2) showed that environmental salinity had no significant effect on death time (0.01%, F-Test). Furthermore, histological observations (2.7.0) failed to reveal any obvious difference in disease manifestations between groups at the various salinities.

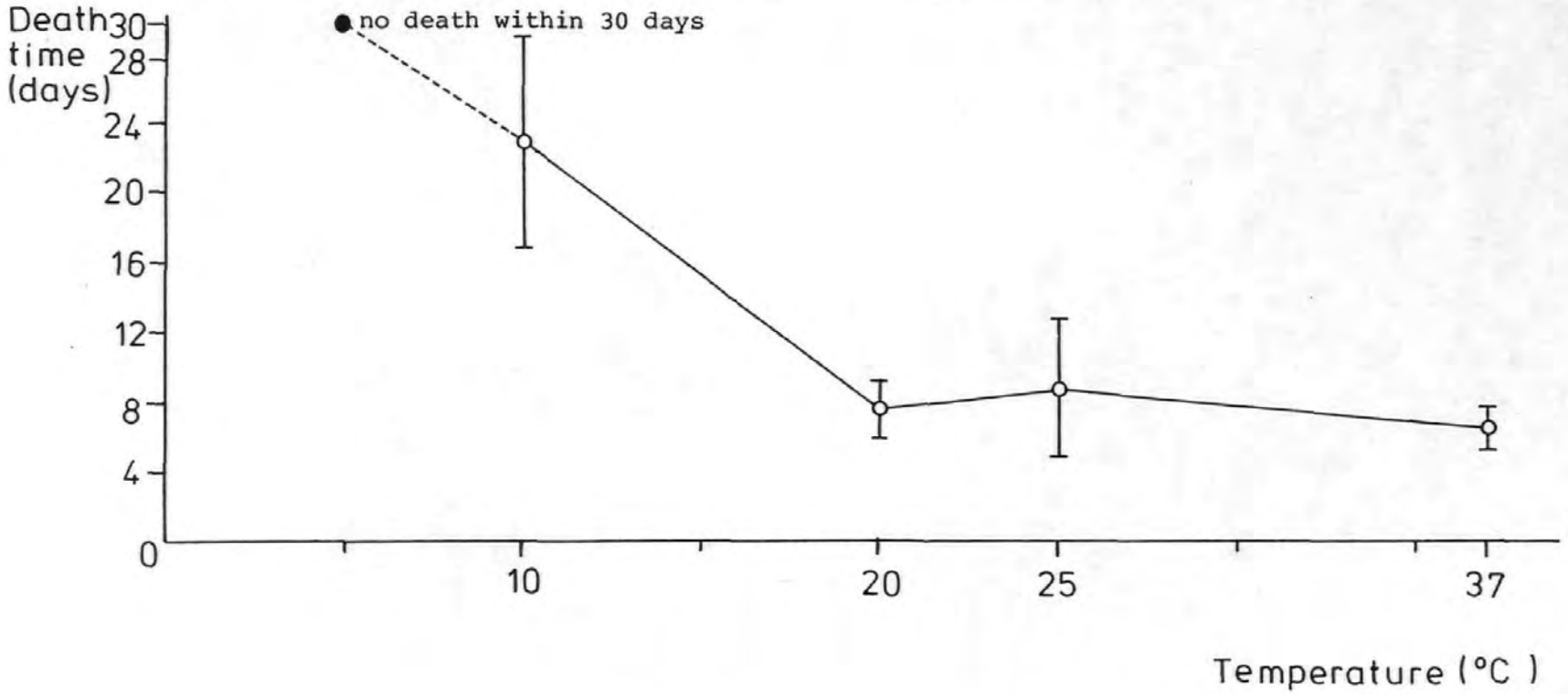
5.2.3 Effect of other environmental influences on pathogenesis

Maintaining eels in water without aeration following experimental infection (2.11.3) was found to make no appreciable difference to death time. Fish deprived of aeration kept to the surface waters of the tanks, whilst changing their respiration from the normal short, frequent buccal pumps to slow, deep buccal pumps.

Eels placed in water deliberately polluted, or placed into full strength sea water (2.11.3) died approximately 30 hr post-inoculation with no significant difference in death time.

5.2.4 Relationship between fish weight and susceptibility to vibriosis

Fig 9 The effect of temperature on death-time following injection with V.anguillarum.



Eels injected with a standard inoculum per unit weight (2.11.4) were found to show no obvious relationship between fish weight and death time. Plotting the data graphically (Fig. 10) and carrying out linear regression suggested no major correlation between weight and death time of fish.

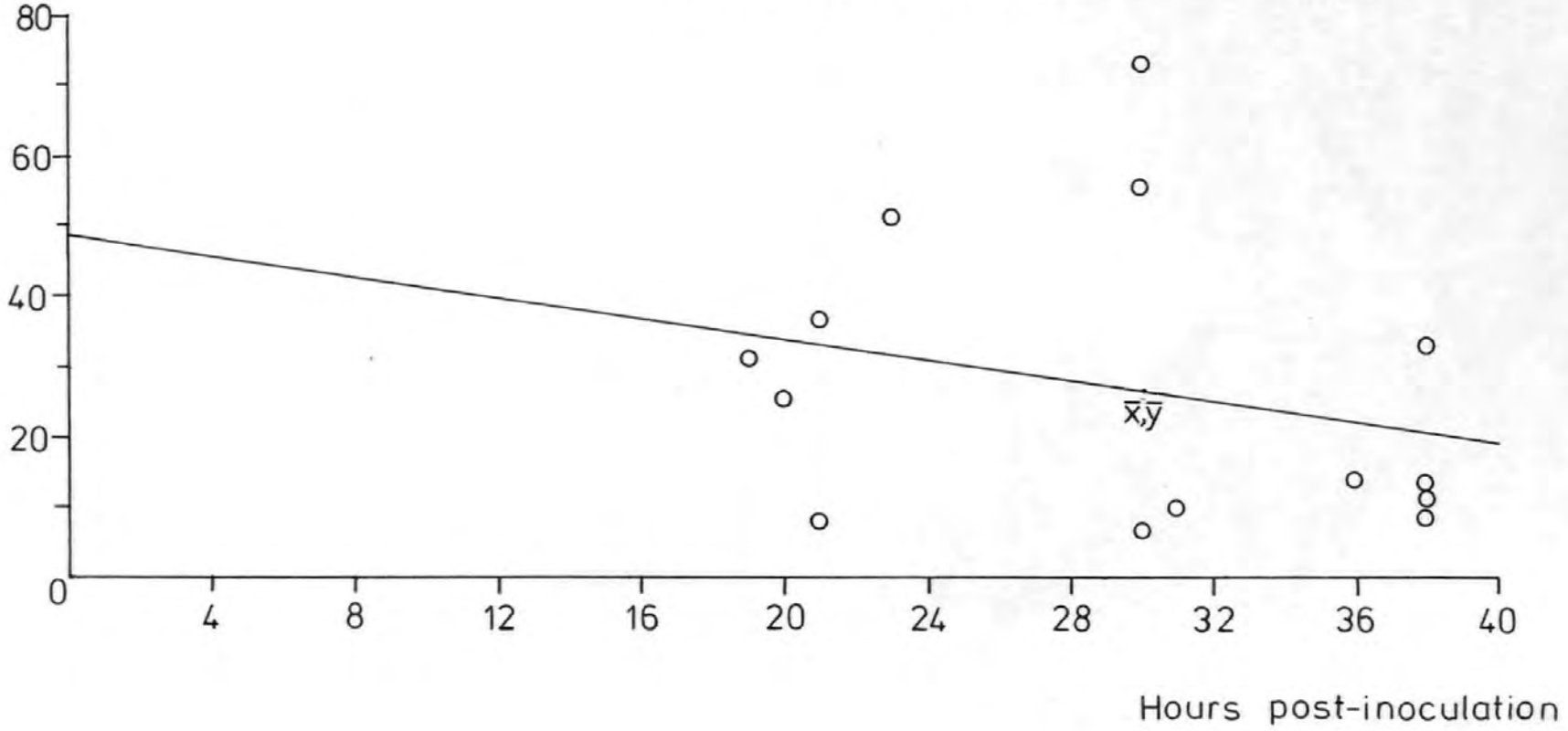
5.3.0 DISCUSSION

From the results it was clear that temperature had a significant effect on vibriosis. Failure to cause disease at 5°C was thought to be due to the slow growth rate of the pathogen at this temperature. In broth culture, *V. anguillarum* multiplies very slowly at 5°C, confirming suppression of growth at this temperature. At higher temperatures fish died more rapidly which was apparently due to the increased growth rate of the organism. Although a rise in environmental temperature leads to an increase in the rate of appearance of an inflammatory response in fish (Finn & Nielson, 1971) the eels would seem unable to cope with the multiplication of the pathogen. Although *V. anguillarum* strain UNH 569 grows poorly at 37°C eels challenged at this temperature died relatively quickly. This was thought to be due to stress caused by the necessity to increase respiratory rate and other physiological compensation mechanisms, in the face of decreased oxygen availability at this higher temperature.

If pathogenesis was directly related to a critical population size, then the organism would produce disease most rapidly at its optimum growth temperature, 25°C. Therefore it would seem that optimum conditions for virulence *in vivo* may not be the same as those for growth *in vitro*. In *Chondrococcus* (*Flexibacter*) *columnaris*, the causative organism of columnaris disease in fish, Pacha and Ordal (1963)

Fig 10 Relationship between weight and death-time for eels injected with V.anguillarum

(line of best fit determined by linear regression)



demonstrated that highly virulent strains were able to produce disease at a lower temperature than less virulent strains, suggesting that pathogenicity can be influenced by temperature. In addition, at 25°C host defence mechanisms could be more effective. In poikilothermic animals resistance to pathogens was increased at temperatures above the preferred normal (Covert & Reynolds, 1977; Kluger et al., 1975). A possible explanation for this is that febrile temperatures caused a reduction in availability of serum iron. V. anguillarum has been shown to produce iron sequestering mechanisms (Crosa & Hodges, 1981) and this may be less efficient at 25°C than at 20°C.

Under intensive culture conditions, outbreaks of vibriosis in fish have been associated with an increase in ambient temperature (Ross, 1970; Fryer et al., 1972; Wood, 1974; Didier, 1974); however in contrast, eels cultured in heated brackish water at Hinkley Point fish farm, contracted vibriosis following a drop in water temperature (Ingram, personal communication). Although temperature appears to be important in the pathogenesis of vibriosis, it remains unknown how temperature-dependent environmental phenomena in natural aquatic ecosystems affect the host-pathogen relationship both in the wild and in cultured fish populations. Fish tend to be cultured in natural waterways or in systems receiving water from natural sources, and so variations in fauna and flora would eventually affect these cultured fish. Increase in temperature in association with increased day length govern the onset of the spring algal blooms. This causes dramatic changes in the availability of nutrients in the populations of zooplankton and subsequently fish stocks. The work of Didier (1974) showed outbreaks of vibriosis were associated with a decrease in water clarity, probably due to algal blooms. Algal blooms contain populations of blue green bacteria (Cyanophyta) which produce toxic

metabolites (Odum, 1971) the effects of which on the host-pathogen interaction have not been considered. Although natural outbreaks of vibriosis were not an integral part of this study, environmental aspects of this nature should be considered.

Environmental salinity had no significant effect on death-time when fish were injected with V. anguillarum. This is in contrast to the earlier observation that entry of the host by the pathogen from the surrounding medium was affected by salinity (4.2.4).

In the present study, influencing eels with potentially stressing conditions did not affect death-time or disease manifestations. Eels have been shown to be resistant to many forms of 'stress' (Larsson & Fänge, 1969) and it may be that the conditions imposed on the eels during experimentation failed to produce stress in these fish. How other, more sensitive, species would have responded to the influences applied remains a topic for further research. Nevertheless, handling and overcrowding stresses have been reported as initiating outbreaks of vibriosis in salmonids (Ross, 1970; Fryer et al., 1972; Wood, 1974; Berjerano & Sarig, 1979).

Deliberate overcrowding of carp following experimental infection with A. punctata, showed these fish to have a higher mortality rate than infected carp in less crowded conditions (Avtalion et al., 1973). Due to high stocking density, excreted ammonia can reach abnormally high levels consequently causing stress (Shepherd & Poupard, 1975).

Copper stress was suggested to be responsible for an outbreak of vibriosis in eels thought to have a latent infection of V. anguillarum (Rødsoether et al., 1977). Heavy metals have been shown to affect the physiology of fish. Kendal (1977) showed that low levels of mercury caused liver and kidney necrosis in catfish; while Stevens (1977) reported similar observations in P. americanus and copper has been

found to be responsible for changes in blood glucose, chloride, protein and haemoglobin (Chistensen et al., 1972). Copper has also been found to affect blood parameters when combined with water 'hardness' and pH fluctuations (Waiwood, 1980).

Fish have been shown to have seasonally altered physiological parameters. Van Vuren and Hattingh (1978) found that blood haemoglobin, haematocrit and blood cell number varies throughout the year. Diadromous species which migrate from one salinity to another have been shown to alter their physiology to suit the new environment. The effect of the environment on both the fish and pathogen appears to be complex and multifactorial requiring a better understanding of these relationships. Stress is in itself a complex phenomenon (Fig. 8) making its relationship with disease susceptibility obscure.

CHAPTER 6. POSSIBLE PATHOGENIC MECHANISMS AND VIRULENCE DETERMINANTS OF V. ANGUILLARUM

6.1.0 INTRODUCTION

For an organism to be pathogenic it has to be able to overcome host defence mechanisms and satisfy nutritional requirements for survival and disease production.

It has only recently been recognised that improvements in the control of a disease may be achieved by an understanding of how the pathogen produces disease manifestations, and once determined seeking to neutralise these pathogenic determinants (Smith, 1974).

Earlier studies demonstrated V. anguillarum to be invasive, causing manifestations of disease in fish (Chapter 3). The present chapter of this dissertation deals with the possible pathogenic mechanisms of V. anguillarum in the eel based on the key aspects of pathogenicity proposed by Smith (1978).

To facilitate comprehension of the discussion for this chapter, data are discussed within six areas dealing with the pathogenicity (6.3.1), infection mechanisms (6.3.3), toxin production (6.3.4), colonial morphology and ultrastructure (6.3.5), envelope proteins and plasmid analysis (6.3.6) and other properties of V. anguillarum and their relationship with virulence in the eel (6.3.2).

6.2.0 RESULTS

6.2.1 Determination of lethal dose 50 (LD-50)

Following initial pathogenicity assessments (2.4.1), the virulence of 24 strains was tested. Eleven were found to have an LD_{50} of less than 10^9 bacteria with the most virulent strains having an LD-50 in the range of 10^6 - 10^7 viable bacteria; while the

remaining strains were considered of low virulence. Of 6 strains assessed for pathogenicity in grey mullet, all were found to be virulent, with LD_{50} values found to be considerably lower than those found in eels. For results of virulence determinations see Table 9.

6.2.2 Enhancement and attenuation of virulence of V. anguillarum

V. anguillarum strain UNH 569 was passaged or repeatedly subcultured (2.12.2). The virulence of the passaged strain was increased by a factor of 10^2, while subculture induced only a slight attenuation of virulence (Table 9).

6.2.3 Fate of virulent and low virulence strains in eels

Results of initial pathogenicity determinations showed a fundamental difference between strains in their ability to survive and cause disease in eels. To elucidate the behaviour of strains in vivo during pathogenesis, eels were injected with a virulent and a low virulent strain and the kinetics of invasion studied (2.12.3). One group of eels received strain UNH 569 (LD 50 = 1.1×10^7) while a second group was inoculated with strain PB-15 (LD-50 > 10^9). Eels receiving the virulent strain were sampled at 6, 12 and 15 hr and tissue bacteria enumerated (2.2.6); the group of eels receiving PB-15 were sampled daily for three days. Viable counts performed on eel tissues from fish injected with UNH 569 demonstrated V. anguillarum in all tissues examined by 6 hr post-inoculation. These bacterial numbers gradually increased over time with largest numbers being found in spleen (Table 10). Eels receiving the low virulence strain, contained approximately 10^3 viable bacteria gm^{-1} one day post-inoculation with numbers decreasing below detection by 3 days post-inoculation (Table 11). In another experiment it was found that

Table 9 Virulence, Plasmid content and Colistin sensitivity of *V.anguillarum*

Strain No	LD50 (eels)	LD50 (Mullet)	Plasmid content (mdal)	MIC of Colistin ($\mu g\ cm^{-3}$)
3022A	2.1×10^7	nd	44	400
UNH 569	1.1×10^7	3.2×10^5	44	400
HC 2	2.1×10^7	1.7×10^4	44	400
PT-479	4.3×10^7	nd	-	0.05
PT-78069	7.6×10^7	nd	-	0.05
A1330/78	1.1×10^8	nd	43	400
8.15	1.1×10^8	nd	-	0.05
PT 78001	3.3×10^8	nd	44,23	50
NCMB 829	3.3×10^8	nd	-	0.05
MS 549	3.4×10^8	6.0×10^4	8,12	0.05
775	4.3×10^8	nd	44	400
NCMB 6	$>10^9$	nd	44,23	50
NCMB 1873	$>10^9$	nd	48	0.25
NCMB 1876	$>10^9$	nd	47	0.05
A20/76	$>10^9$	nd	45	50
A555/76	$>10^9$	nd	44	650
1669	$>10^9$	nd	49	600
COB 408	$>10^9$	7.2×10^6	48	0.25
34/71	$>10^9$	nd	46	50
7/75	$>10^9$	4.3×10^6	58	0.25
MS 2072	$>10^9$	nd	-	0.25
PB-15	$>10^9$	1.4×10^6	48	0.25
PT-7601	$>10^9$	nd	45	0.05
NCMB 407	$>10^9$	nd	46	400
NCMB 571	nd	nd	46	400
NCMB 572	nd	nd	45	400
NCMB 1291	nd	nd	44	0.05
NCMB 1336	nd	nd	49	400
8.13	nd	nd	48	400
8.14	nd	nd	49	650
15/75	nd	nd	47	400
MS424	nd	nd	46	400
MS439	nd	nd	47	400
MS463	nd	nd	47	650
MS469	nd	nd	44	650
MS974	nd	nd	46	25
PO-103	nd	nd	48	400
UNH 569 (passaged)	1.9×10^5	nd	nd	nd
UNH 569 (attenuated)	6.6×10^7	nd	nd	nd

nd= not determined.

Table 10 Viable bacteria gm^{-1} tissue sampled at 6,12 & 15hr following inoculation with the virulent strain:UNH 569

Organ	Viable bacteria gm^{-1}		
	6hr	12hr	15hr
spleen	$6.7 \times 10^{7}_{7}$	$6.3 \times 10^{8}_{7}$	$2.3 \times 10^{10}_{9}$
heart	$1.7 \times 10^{7}_{7}$	$5.0 \times 10^{7}_{8}$	$1.5 \times 10^{8}_{8}$
Kidney	$1.9 \times 10^{6}_{6}$	$1.4 \times 10^{7}_{7}$	$4.5 \times 10^{7}_{7}$
liver	$5.1 \times 10^{6}_{6}$	$4.1 \times 10^{7}_{6}$	$4.9 \times 10^{7}_{8}$
gut	$1.1 \times 10^{6}_{6}$	$7.8 \times 10^{6}_{6}$	$4.5 \times 10^{8}_{8}$

Table 11 Viable bacteria gm^{-1} tissue sampled at 1,2 & 3 days following inoculation with the low-virulence strain PB-15

Organ	Viable bacteria gm^{-1}		
	1	2	3
spleen	$9.8 \times 10^{3}_{3}$	<50	<50
kidney	$3.6 \times 10^{3}_{3}$	$5.4 \times 10^{2}_{2}$	<50
liver	$2.5 \times 10^{3}_{3}$	$1.4 \times 10^{2}_{2}$	<50

injection of virulent and low virulence strains caused haemosiderin to be deposited in the liver (3.2.10).

6.2.4 In vitro phagocytosis of V. anguillarum strains by eel macrophages

To investigate the fish phagocyte-pathogen relationship, the virulent strain (UNH 569) was compared to a low virulence strain (PB-15) in their ability to avoid phagocytosis. Fluoresceinated bacteria (2.12.5) were mixed with eel phagocytes in tissue culture plates containing Eagle's medium (2.12.4) to give a bacterium: phagocyte ratio of about 100:1. After 2 hr, the reaction was stopped by the addition of methanol followed by staining with Giemsa and examination by light and ultraviolet microscopy, revealing that both strains were actively taken-up by these phagocytes. Quantitative uptake was not assessed.

6.2.5 Response of V. anguillarum to natural agglutinating antibody in eel plasma

The possibility of virulent strains of V. anguillarum being able to avoid being agglutinated by natural agglutinating antibody present in eel plasma was assessed (2.12.6). Virulent strains (UNH 569, HC2, 3022A and MS 549) were compared to low virulence strains (7/75, NCMB6, COB 408, 1669 and PB-15). Strains UNH 569, HC2, MS 549, 7/75, NCMB6, 1669 and PB-15 were all agglutinated by eel plasma and 3022A and COB 408 were not.

6.2.6 Ability of V. anguillarum to agglutinate erythrocytes

Comparison between virulent and low virulence strains of V. anguillarum with respect to their ability to cause haemagglutination was assessed using guinea-pig, rabbit and eel erythrocytes (2.12.7).

Of 11 strains, 8 caused agglutination of rabbit and guinea-pig red blood cells, while none of the strains agglutinated eel erythrocytes (Table 12).

Galactose, maltose and fucose had no effect on haemagglutination but, the addition of mannose inhibited agglutination caused by MS 549 and 7/75.

6.2.7 Effect of fish bile on V. anguillarum

The ability of virulent and low-virulence strains of V. anguillarum to grow in the presence of bile was investigated using eel, plaice and carp bile (2.12.8). It was found that all strains, regardless of virulence status, could grow in the presence of these fish bile types.

6.2.8 Effect of V. anguillarum cell wall material in vivo

To elucidate the effect of cell wall material on eels, fish were injected ip (2.12.9). Administration of sonicated preparations (from approximately 10^8 viable bacteria), failed to produce mortality or disease manifestations of vibriosis; although haemosiderin was observed in liver tissues, 3 hr post-injection.

6.2.9 Effect of extracellular products of V. anguillarum in vivo

Toxicity of cell-free culture supernatant was determined in eels (2.12.10). Injections (ip) of 0.1 cm^3 of preparation failed to produce death or visible signs of disease at the injection site. Examination of this location by histology (2.7.0) revealed slight cellular infiltration of adjacent tissues one day post-injection; however, one week post-injection there were no noticeable tissue changes.

Table 12 Haemagglutination properties of V.anguillarum and their relationship with virulence

		Erythrocytes:	Rabbit/ guinea-pig				Eel	
Strain	LD-50	- sugar	+ mannose	+ fucose	+ galactose	+ maltose	- sugar	+ sugar
UNH 569	1.1×10^7	+	+	+	+	+	-	-
UNH 569 (attenuated)	6.6×10^7	+	+	+	+	+	-	-
HC 2	2.1×10^7	+	+	+	+	+	-	-
3022A	2.1×10^6	-	-	-	-	-	-	-
MS 549	3.4×10^8	+	-	+	+	+	-	-
HC 7	*	-	-	-	-	-	-	-
7/75	$>10^9$	+	-	+	+	+	-	-
NCMB 6	$>10^9$	+	+	+	+	+	-	-
COB 408	$>10^9$	-	-	-	-	-	-	-
1669	$>10^9$	+	+	+	+	+	-	-
PB-15	$>10^9$	+	+	+	+	+	-	-

*-strain isolated from outbreak of vibriosis

6.2.10 Qualitative and quantitative haemolysin production by *V. anguillarum*

To examine the production of haemolysin by *V. anguillarum*, strains were cultured on blood agar (2.12.11). Most strains showed β-haemolysis on horse, eel and plaice blood agar, while strains 7/75 (Plate 30b) and 1669 (Plate 27a) were only weakly haemolytic. Haemolysis was of two types designated : types I and II (Fig. 11, Plate 27a). Type I haemolysis appeared as a clear lytic zone surrounding the bacterial colony (Plate 27a); while type II haemolysis appeared as a clear zone, surrounded by a zone of partial lysis (Plate 27a). Electron microscopy of agar blocks taken from the clear zone revealed erythrocyte 'ghosts' with apparently intact cell membranes surrounding cytoplasm devoid of electron dense material (Plate 27b). The partially lysed zone contained both lysed and intact erythrocytes.

Comparisons of strains of varying virulence for haemolytic ability, showed some strains to be haemolytic (Table 13); while other strains were weakly haemolytic (Plates 28-30). Isolates of the organism were found to be very haemolytic when freshly isolated from infected fish; furthermore, it was found that subcultured strains (2.12.2) had reduced haemolysin production (Plate 31).

A measure of haemolysin production was obtained by calculating the ratio between colony and lytic zone diameters (2.12.11). Daily measurements (Table 13) showed the kinetics of haemolysis to have three forms: i) strains 3022A, 8.15, 775 and PB-15 caused haemolysis at an almost constant rate over time; ii) strains HC2, HC7, COB 408 and MS 2072 caused initially large amounts of haemolysis which decreased with time, while iii) strains UNH 569, MS 549 and NCMB6 gradually increased haemolytic activity over the 5 day period.

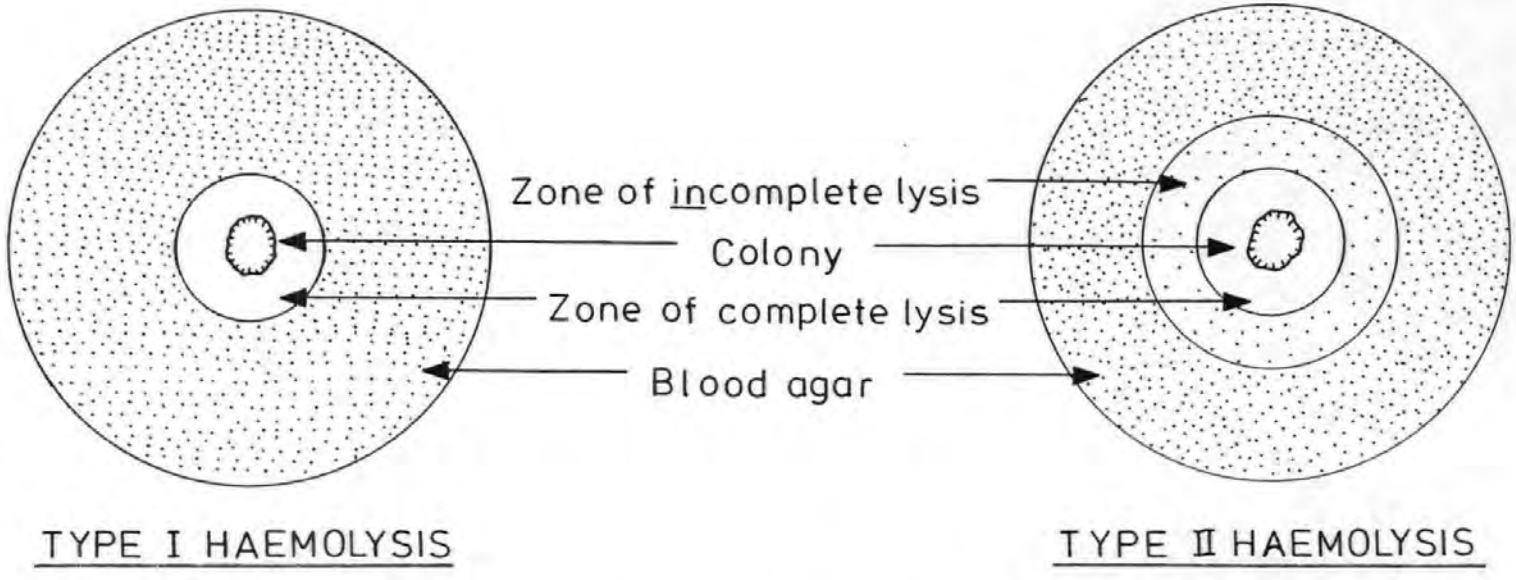


Fig 11 Diagrammatic representation of types of haemolysis on horse blood agar.

Table 13 Haemolytic properties and colonial morphology of V.anguillarum and their relationship with virulence in eels.

Strain No	LD 50	Colonial morphology[1]		Haemolysis type[2]	Haemolysis production (hr)[3]			
					48	72	96	120
3022A	2.2×10^6	s	o	I	2.12	2.01	1.90	1.80
UNH 569	1.1×10^7	s	c	I	1.00	1.21	1.50	1.90
HC 2	2.1×10^7	l	c	II	2.00	1.64	1.28	1.60
8.15	1.1×10^8	s,l	c	I	1.00	1.09	1.00	1.00
MS 549	3.4×10^8	s,l	c	I	1.60	1.46	1.20	2.00
775	4.3×10^8	s	c	I	1.00	1.00	1.00	1.00
HC 7	*	s	c	I	2.00	1.87	1.80	1.74
7/75	$>10^9$	s	c	weakly haemolytic				
NCMB 6	$>10^9$	s,l	c	II	1.36	1.17	1.60	1.90
COB 408	$>10^9$	l	c	II	1.80	1.43	1.20	1.90
1669	$>10^9$	s,l	c	weakly haemolytic				
MS 2072	$>10^9$	s,l	c	I	1.67	1.25	1.30	1.40
PB-15	$>10^9$	s,l	c	II	2.10	1.90	2.10	2.10

[1] s = small, l = large, o = opaque, c = clear

[2] I = single zone of haemolysis, II = single zone of haemolysis plus 'halo' of partial haemolysis.

[3] Haemolytic ability assessed daily, given as the ratio : zone diameter/colony diameter.

* Strain of V.anguillarum isolated from natural outbreak in eels.

6.2.11 Effect of a crude haemolysin preparation in vivo

The role of V. anguillarum haemolysins in pathogenesis in eels was investigated (2.12.10). Intravascular (iv) injection with a crude haemolysin preparation (2.12.10). (300 haemolytic units, HU) caused anaemia in eels (treated = 24.1 ± 1.84%, Control = 32.95 ± 1.3%), producing a significant decrease in haematocrit (at both 0.05 and 0.01 levels, F-test). Although death did not occur, small petechial haemorrhages were present on the ventral surfaces and 20 hr post-injection and dissection of decapitated eels showed the liver and spleen to be pale and haemorrhaged. Histological observations revealed haemorrhaging and cellular damage of spleen, kidney and liver; however, the intestinal mucosa was unaffected and haemosiderin was detected in liver sections.

Injection (iv) with a more concentrated haemolysin preparation (1000 HU) caused rapid death characterised by flaccidity of body musculature and haemolysis; no external signs of disease were present.

Haemolysin preparations, boiled for 5 min, and injected (iv) failed to produce death or visible signs of disease in eels.

6.2.12 Detection of V. anguillarum haemolysin in vivo

To ascertain whether V. anguillarum produces haemolysin(s) during pathogenesis, plasma from infected eels were analysed for the presence of haemolysin using crossed- and rocket-immuno-electrophoresis (2.12.12). Haemolysin could not be detected by the techniques applied however, analysis of crude haemolysin preparation (2.12.10) by rocket immuno-electrophoresis produced readily distinguished rockets when the samples were suitably diluted.

6.2.13 Enzyme Production by V. anguillarum

Culturing strains of V. anguillarum on media with specific substrates (2.12.13) demonstrated that V. anguillarum produced a range of enzymes (Table 14). All strains utilized Tween 80 (polyoxyethylene-sorbitan-mono-oleate), while none of the strains utilized tributyrin.

A well defined reaction was observed on elastin agar; strains either producing large amounts of this enzyme or showing no activity at all (Table 14).

A measure of enzyme production was obtained by placing cell-free culture supernatant from a 20 hr broth culture into wells cut into agar plates containing specific substrates and measuring the zones of breakdown following incubation (25°C, 24 hr). All strains produced DNase and amylase, however not all strains produced protease, lipase and coagulase enzymes (Table 14). To differentiate between lipase and coagulase activity, fatty acids produced by lipolytic activity were precipitated with calcium salts and converted to lead salts producing a brown-black deposit readily discernible from areas of coagulase activity. All strains produced coagulase except COB 408, MS 2072 and PB-15, while lipase was not produced by 1669 (Table 14).

Strains UNH 569, HC2, MS 549 and their respective substrains and 3022A; appeared to have reduced DNase activity in the presence of calcium ions. Attenuated substrains showed generally less DNase activity while on other substrates the response was variable (Table 14).

6.2.14 Quantitative protease production by V. anguillarum

Examination of V. anguillarum broth culture supernatants for proteases (2.12.13) showed this organism to produce only trace amounts of these enzymes under the culture conditions employed.

Table 14 Enzyme production by V.anguillarum and its relationship with virulence in eels

Strain	LD 50	Cell free supernatant[1] DNase -Ca	DNase +Ca	Protease (milk)	Lipase (egg reaction)	Coagulase	Amylase	Plate culture Lipase Tween 80	Lipase Tributyrin	Elastase
3022A	2.1×10^6	13	12	21	8	18	nd	+	-	-
UNH 569	1.1×10^7	14	9	17	8	20	8	+	-	-
UNH 569 (attenuated)	6.6×10^7	14	10	14	9	13	7	+	-	-
HC 2	2.1×10^7	13	10	17	11	20	9	+	-	-
HC 2 (attenuated)	nd	12	9	19	10	21	10	+	-	-
MS 549	3.4×10^8	17	13	17	9	16	9	+	-	+
MS 549 (attenuated)	nd	15	14	18	9	21	12	+	-	+
COB 408	$>10^9$	12	7	10	9	0	7	+	-	-
1669	$>10^9$	7	7	0	0	14	7	+	-	-
MS 2072	$>10^9$	12	12	0	10	0	14	+	-	nd
PB-15	$>10^9$	16	16	16	10	0	7	+	-	+

1=zone diameter in agar plate following incubation with $0.1cm^3$ cell free TSB culture supernatant
nd = not determined.

6.2.15 Elucidation of enzyme production in vivo by histochemistry

Histochemical analysis of infected and control tissues from routinely infected eels revealed little change in PAS, AB and Sudan Black positive material (2.12.14). Infected tissues stained with PAS, clearly demonstrated mucous cells in dislodged intestinal epithelium. Both control and infected tissues stained weakly with alcian blue and sudan black. Tissues were frequently altered by the influx of erythrocytes indicative of haemorrhage (Plate 32) making histochemical observations difficult.

6.2.16 Possible capsule production by V. anguillarum

Electron lucent zones observed around tissue bacteria by electron microscopy (Plate 33) were examined for possible capsular properties by staining with ruthenium red (2.7.9). Tissue, broth and agar-cultured strains failed to give a positive capsule staining reaction (Plate 34a) when compared to a known capsulated strain of Klebsiella pneumoniae, the capsule of which was evident with and without staining (Plate 34b & c). Capsular material was undetectable by staining and observation by light microscopy (2.7.3).

6.2.17 Examination of colonial morphology with respect to virulence in V. anguillarum

Examination of V. anguillarum strains cultured on agar (2.12.15) showed colonies to be shiny, domed and round, and either clear or opaque. Size of colony tended to be strain specific although some strains produced both large and small colonies (Table 13). Attenuated substrains of UNH 569, HC2 and MS 549 developed umbonate colonies as opposed to the typical domed colonies of parent strains (Plate 35).

6.2.18 The ultrastructure of V. anguillarum

Ultrastructural examination of V. anguillarum strains of varying virulence in eels, was carried out (2.12.16).

Negatively stained and metal-shadowed preparations of V. anguillarum strains established that this species had two distinct cell forms. Bacteria were either long and thin, and straight or slightly curved (1.8 x 0.46 µm by negative staining or 2.84 x 0.97 µm by metal shadowing) (Plate 35), or shorter and oval (1.2 x 0.82 µm by negative staining or 1.5 x 0.91 µm by metal shadowing) (Plate 36).

Bacteria stained by heavy metal salts appeared to have a rugose outer membrane (Plate 36) which was evident in V. anguillarum obtained from infected fish tissues (Plate 38b & c). Bacteria were found to have 1, 2 or 3 flagella located at the pole of the cell (Plate 36), the multiflagellate bacteria having both long and short cellular morphology (Plate 37). It was apparent that some strains produced more multiflagellate individuals than others (e.g. HC2 Plate 38a) however, multiflagellate bacteria were only observed in virulent strains.

Flagella measured approximately 30 nm in diameter and were 2-3 times the length of the bacterial cell. Metal-shadowed preparations of damaged flagella revealed this structure to consist of a central core surrounded by an outer sheath (Plate 39a). Intact flagella were found to have a spherical swelling at the terminus, approximately 40 nm in diameter (Plate 39b).

Metal-shadowed and negatively stained preparations demonstrated the occasional presence of pili (approximately 7.5 nm diameter) in some strains; however pilated forms were infrequently encountered (Plate 40a & b). Metal-shadowed preparations also revealed the occasional presence of cytoplasmic inclusions (1-3 per cell) appearing

as 'humps' on gold-palladium electronmicrographs (Plate 41).

Examination of ultrathin sections of bacteria revealed two types of bacterial envelope structure, which were dependent on culture conditions. Bacteria cultured in agar appeared to have a typical gram-negative envelope of a multilayered topography (Plate 42a). Bacteria cultured in broth or observed in diseased tissues appeared to have the cytoplasmic membrane surrounded by a wavy, loose-fitting outer membrane (Plate 42b). Detailed observations revealed both envelope types to have a five layered outer membrane, a distinct periplasmic space and a cytoplasmic membrane. Dimensions of the envelope layers, labelled after De Petris (1967) are shown in Fig. 12.

6.2.19 Envelope and outer membrane proteins of V. anguillarum

Virulent and low virulence strains of V. anguillarum were examined for differences in envelope biochemistry by electrophoretically separating constituent proteins (2.8.1) obtained from sphaeroplasts (2.12.17) and chemically extracted cell wall components (2.12.19 - 2.12.22). Examination of outer membrane and whole envelope preparations (2.12.18) gave information on the location of major proteins.

Examination of envelope protein profiles of broth-cultured bacterial sphaeroplasts demonstrated that the majority of strains contained a major envelope protein ranging from 28700 - 31200 daltons (Plate 43b). Strain MS 2072 contained several smaller proteins as opposed to a single major protein, while strain PB-15 contained a 33200 dalton protein in addition to a 30500 dalton protein. Strain HC7 (not illustrated) as opposed to having a single major protein, had four proteins of 29500, 33700, 36000 and 72000 daltons.

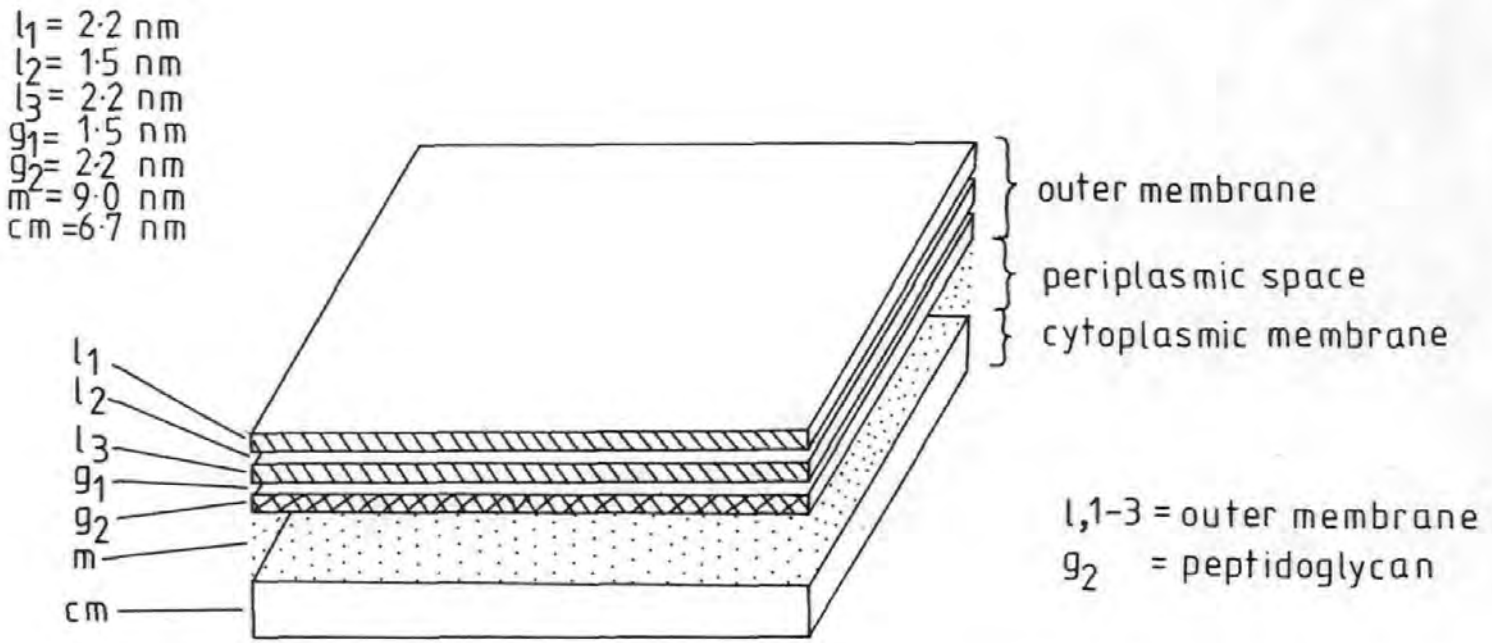


Fig 12 Diagrammatic representation of the V.anguillarum cell envelope giving approximate dimensions of constituent layers(labelled after De Petris,1967).

Protein profiles of sphaeroplasts from bacteria cultured on agar (10 µg loaded onto gels) closely resembled the profiles of broth-cultured sphaeroplasts (Plate 44a); application of smaller protein quantities (5 µg) gave better resolution for observing the major envelope proteins (Plate 44b), which had a molecular weight range of 29500 to 31200 daltons. It was shown that the extra membrane protein of strain PB-15 had a molecular weight of 35000 daltons and strain MS 2072 possessed four major proteins of molecular weight, 30000, 33000, 33500 and 36000 daltons. Sphaeroplast formation was checked by electron microscopy.

Extraction by guanidine hydrochloride (Plate 43a) gave similar profiles, but more bands than, those obtained from broth-cultured sphaeroplasts, furthermore the major proteins ranged in molecular weight from 34000 to 36000 daltons. Strain MS 2072 was found to have several major proteins, while strain PB-15 had an additional protein of 38000 daltons.

Electrophoretic separation of envelope proteins extracted by lithium acetate (Plate 45a) revealed that this technique isolated relatively fewer proteins than observed in envelope sphaeroplasts. The major proteins had a molecular weight range of 28000 to 30000 daltons, with the profile of MS 2072 having a single major protein of 30000 daltons. In addition, by this extraction technique, all strains possessed proteins of 33000, 42000 and 49000 daltons.

Profiles of proteins extracted using Triton X-100 (2.12.20) demonstrate major proteins with a molecular weight range of 29000 to 31000 daltons (Plate 45b). Single major proteins were absent from strain MS 2072, while strain PB-15 had an additional protein of 34000 daltons.

Treating bacterial envelope sphaeroplasts with sarkosyl

(2.12.18), dissolved the cytoplasmic membrane leaving the outer membrane intact. Separation of outer membrane and whole envelope proteins revealed the major envelope proteins to be situated in the outer membrane (Plate 46a). In addition to the major protein, five other proteins of molecular weight 14200, 22700, 27000, 31500 and 39000 daltons were also present in the outer membrane.

Envelope proteins from strain UNH 569 and its attenuated substrains appear to be identical in their profiles (Plate 46b).

Following chemical extraction of envelope proteins, treated bacterial cells were examined by electron microscopy to assess the extent of the extraction procedure. Bacteria extracted using Triton X-100 (Plate 47) and lithium acetate appeared to lose the outer membrane while maintaining cellular integrity. However, cells treated with guanidine hydrochloride showed large holes present in the cell wall giving the bacteria the appearance of a sponge.

Comparisons between protein profiles of virulent and low virulence strains (Table 15) did not reveal any statistically significant correlation between the major outer membrane proteins and virulence (one-way ANOVA).

6.2.20 Extraction and separation of *V. anguillarum* plasmid DNA

Plasmid DNA was extracted from 37 strains of *V. anguillarum* (2.12.25). Of the 37 strains examined, 31 were found to contain a large plasmid (mean molecular weight 47 megadaltons, determined by comparison of migration distances of 5.6, 36, 62 and 72 megadalton plasmids of *E. coli*). In one strain (MS 469) two small plasmids of 5 and 8 megadaltons were observed and two strains carried 23 megadalton plasmids (Table 9).

Table 15 Major outer membrane proteins of V.anguillarum extracted using various procedures

Strain	Sphaeroplasts (broth cultured)	Sphaeroplasts (agar cultured)	Guanidine-HCl	Lithium acetate	Triton-X 100
UNH 569	28700 dal	29500 dal	35000 dal	28000 dal	29000 dal
3022A	28700 dal	29500 dal	34000 dal	nd	30000 dal
MS 549	30000 dal	31000 dal	35500 dal	29500 dal	30500 dal
HC 7	72000,36000 33700,29500	nd	nd	nd	nd
HC 2	31200 dal	31200 dal	nd	nd	31000 dal
COB 408	31200 dal	31200 dal	36000 dal	nd	31000 dal
MS 2072	35200,32000 31000,29500	36000,33500 33000,30000	numerous	30000 dal	nd
PB-15	30500,33200	35000,31000	35000,38000	29000 dal	34000,30500

6.2.21 Antibiotic resistance by V. anguillarum and its relationship with virulence

The possibility that V. anguillarum virulence factors may be genetically linked to resistance/sensitivity to a given antibiotic was examined (2.12.26). All strains were found to be resistant to Multodiscs impregnated with penicillin (2.5 µg) and Cloxacillin (5 µg); although strains were resistant to ampicillin (2 µg) only UNH 569 was resistant to ampicillin at higher concentrations (25 µg) (Tables 16-20). Most strains were sensitive to sulphamethoxazole/trimethoprim (25 µg), tetracycline (10 & 50 µg), streptomycin (10 & 50 µg), gentamycin (10 µg) and nalidixic acid (30 µg). All strains were sensitive to nitrofurantoin (200 µg) except UNH 569.

The in vivo cultured sub strain of UNH 569 was sensitive to sulphamethoxazole/trimethoprim, sulphadiazine, sulphafurazole, nitrofurantoin and chloramphenicol. In contrast, the attenuated substrain was found to be resistant to all these antibiotics. Furthermore, there was a decrease in sensitivity to tetracycline (10 & 50 µg) and sulphafurazole (500 µg). Although similar responses were observed in strains HC2 and MS 549, there were instances where in vivo culture induced resistance when compared to their respective sub strain.

Statistical analysis of antibiotic sensitivity exhibited by in vivo culture and attenuated substrains of UNH 569, demonstrated a significant difference in antibiotic sensitivity ($P = 0.01$). Intra-strain differences in sensitivity were not significantly different for strains HC2 and MS 549 ($P = 0.05$).

No significant difference could be detected between virulent and low virulence strains with regard to their overall antibiotic sensitivity ($P = 0.05$).

Initial Multodisc investigations suggested a correlation between

Table 16 Antibiotic sesitivity of V.anguillarum strain UNH 569

Antibiotic (µg)	Cultured in vivo A[1]	B[2]	Parent strain A	B	Attenuated sub strain A	B
Sulphamethoxazole/ Trimethoprim (25)	30.5	100	28.0	66	R[3]	100
Sulphadiazine (300)	36.0	50	29.0	66	R	100
Sulphafurazole (100)	36.0	50	R	75	R	100
Sulphafurazole (500)	28.0	100	27.0	75	16.0	50
Nitrofurantoin (200)	36.0	100	R	57	R	66
Ampicillin (2)	R	100	R	100	R	100
Ampicillin (25)	18.5	100	R	57	R	66
Penicillin (2.5)	R	100	R	100	R	100
Cloxacillin (5)	R	100	R	100	R	100
Tetracycline (10)	32.0	100	23.0	100	14.0	100
Tetracycline (50)	36.0	100	28.0	100	23.0	100
Chloramphenicol (10)	32.0	100	31.0	50	24.0	50
Chloramphenicol (50)	36.0	100	30.0	57	R	66
Streptomycin (10)	16.0	100	14.0	100	11.0	50
Streptomycin (25)	20.0	100	22.0	100	30.0	50
Gentamycin (10)	30.0	100	30.0	100	26.0	100
Nalidixic acid (30)	36.0	100	27.0	100	31.0	100
Erythromycin (10)	17.0	100	19.0	50	20.0	50

1= mean inhibition zone diameter (mm).
2= percentage of data giving the result in column A.
3= R= resistant to given antibiotic.

Table 17 Antibiotic sensitivity of *V.anguillarum* strain HC 2

Antibiotic (µg)	Parent strain A[1]	Parent strain B[2]	Subcultured derivative A	Subcultured derivative B
Sulphamethoxazole/ Trimethoprim (25)	28.0	100	20.0	100
Sulphadiazine (300)	R[3]	100	R	100
Sulphafurazole (100)	R	100	R	100
Sulphafurazole (500)	13.0	100	11.0	100
Nitrofurantoin (200)	31.0	100	27.0	100
Ampicillin (2)	R	100	R	100
Ampicillin (25)	15.0	66	18.0	100
Penicillin (2.5)	R	100	R	100
Cloxacillin (5)	R	100	R	100
Tetracycline (10)	27.0	100	R	100
Tetracycline (50)	33.0	100	33.0	100
Chloramphenicol (10)	28.0	100	25.0	100
Chloramphenicol (50)	36.0	100	36.0	100
Streptomycin (10)	21.0	100	13.0	100
Streptomycin (25)	24.0	100	23.0	100
Gentamycin (10)	30.0	100	36.0	100
Nalidixic acid (30)	36.0	100	36.0	100
Erythromycin (10)	16.0	100	15.0	100

1= mean inhibition zone diameter (mm).
2= percentage of data giving the result in column A.
3= R= resistant to given antibiotic.

Table 18 Antibiotic sensitivity of *V.anguillarum* strain MS 549

Antibiotic (µg)	Cultured in vivo A[1]	Cultured in vivo B[2]	Parent strain A	Parent strain B	Subcultured derivative A	Subcultured derivative B
Sulphamethoxazole/ Trimethoprim (25)	36.0	100	36.0	100	30.0	100
Sulphadiazine (300)	R[3]	100	32.0	100	20.0	100
Sulphafurazole (100)	R	100	28.0	66	16.0	50
Sulphafurazole (500)	R	100	27.0	100	14.0	50
Nitrofurantoin (200)	24.0	100	28.8	100	26.0	100
Ampicillin (2)	R	100	R	100	R	100
Ampicillin (25)	26.0	50	17.0	100	20.0	100
Penicillin (2.5)	R	100	R	100	R	100
Cloxacillin (5)	R	100	R	100	R	100
Tetracycline (10)	10.0	100	30.0	100	25.0	100
Tetracycline (50)	22.0	100	36.0	100	36.0	100
Chloramphenicol (10)	26.0	100	31.0	100	28.0	100
Chloramphenicol (50)	36.0	100	36.0	100	33.0	100
Streptomycin (10)	nd	-	22.0	66	17.0	100
Streptomycin (25)	22.0	100	23.0	100	19.0	100
Gentamycin (10)	18.0	100	32.0	100	24.0	100
Nalidixic acid (30)	36.0	100	36.0	100	36.0	100
Erythromycin (10)	18.0	100	15.0	100	15.0	100

1= mean inhibition zone diameter (mm)
2= percentage of data giving the result in column A
3= R = resistant to given antibiotic

Table 19 Antibiotic sensitivity by *V.anguillarum* strains 7/75 & COB 408

Antibiotic (µg)	7/75 A[1]	7/75 B[2]	COB408 A	COB408 B
Sulphamethoxazole/ Trimethoprim (25)	36.0	100	22.0	100
Sulphadiazine (300)	30.0	100	R[3]	100
Sulphafurazole (100)	20.0	50	R	100
Sulphafurazole (500)	19.0	100	10.0	50
Nitrofurantoin (200)	32.0	100	27.0	100
Ampicillin (2)	R	100	R	100
Ampicillin (25)	17.0	100	18.0	100
Penicillin (2.5)	R	100	R	100
Cloxacillin (5)	R	100	R	100
Tetracycline (10)	25.0	100	16.0	100
Tetracycline (50)	31.0	100	20.0	100
Chloramphenicol (10)	27.0	100	23.0	100
Chloramphenicol (25)	36.0	100	30.0	100
Streptomycin (10)	23.0	100	14.0	100
Streptomycin (25)	25.0	100	24.0	100
Gentamycin (10)	30.0	100	30.0	100
Nalidixic acid (30)	36.0	100	36.0	100
Erythromycin (10)	19.0	100	14.0	100

1= mean inhibition zone diameter (mm)
2= percentage of data giving the result in column A
3= R = resistant to given antibiotic

Table 20 Antibiotic sensitivity of V.anguillarum strains 1669 & PB-15

Antibiotic (ug)	1669 A[1]	1669 B[2]	PB-15 A	PB-15 B
Sulphamethoxazole/ Trimethoprim (25)	36.0	100	36.0	100
Sulphadiazine (300)	R[3]	100	24.0	100
Sulphafurazole (100)	R	100	27.0	100
Sulphafurazole (500)	12.0	50	31.0	100
Nitrofurantoin (200)	28.0	100	29.0	100
Ampicillin (2)	R	100	R	100
Ampicillin (25)	18.0	66	17.0	100
Penicillin (2.5)	R	100	R	100
Cloxacillin (5)	R	100	R	100
Tetracycline (10)	24.0	100	29.0	100
Tetracycline (50)	32.0	100	29.0	100
Chloramphenicol (10)	21.0	100	31.0	100
Chloramphenicol (50)	36.0	100	36.0	100
Streptomycin (10)	14.0	100	19.0	100
Streptomycin (25)	17.0	100	23.0	100
Gentamycin (10)	30.0	100	25.0	100
Nalidixic acid (30)	36.0	100	25.0	100
Erythromycin (10)	21.0	100	21.0	100

1= mean inhibition zone diameter (mm).
2= percentage of data giving the result in column A
3= R =resistant to given antibiotic

colistin resistance and possession of a plasmid of approximately 47 megadaltons. Minimum inhibitory concentrations (MIC), clearly demonstrated a link between colistin resistance and plasmid possession (Table 9); with all strains having colistin resistance containing the 47 megadalton plasmid, but not all plasmid-carrying strains were resistant.

6.2.22 Relationship between pH tolerance and virulence in *V. anguillarum*

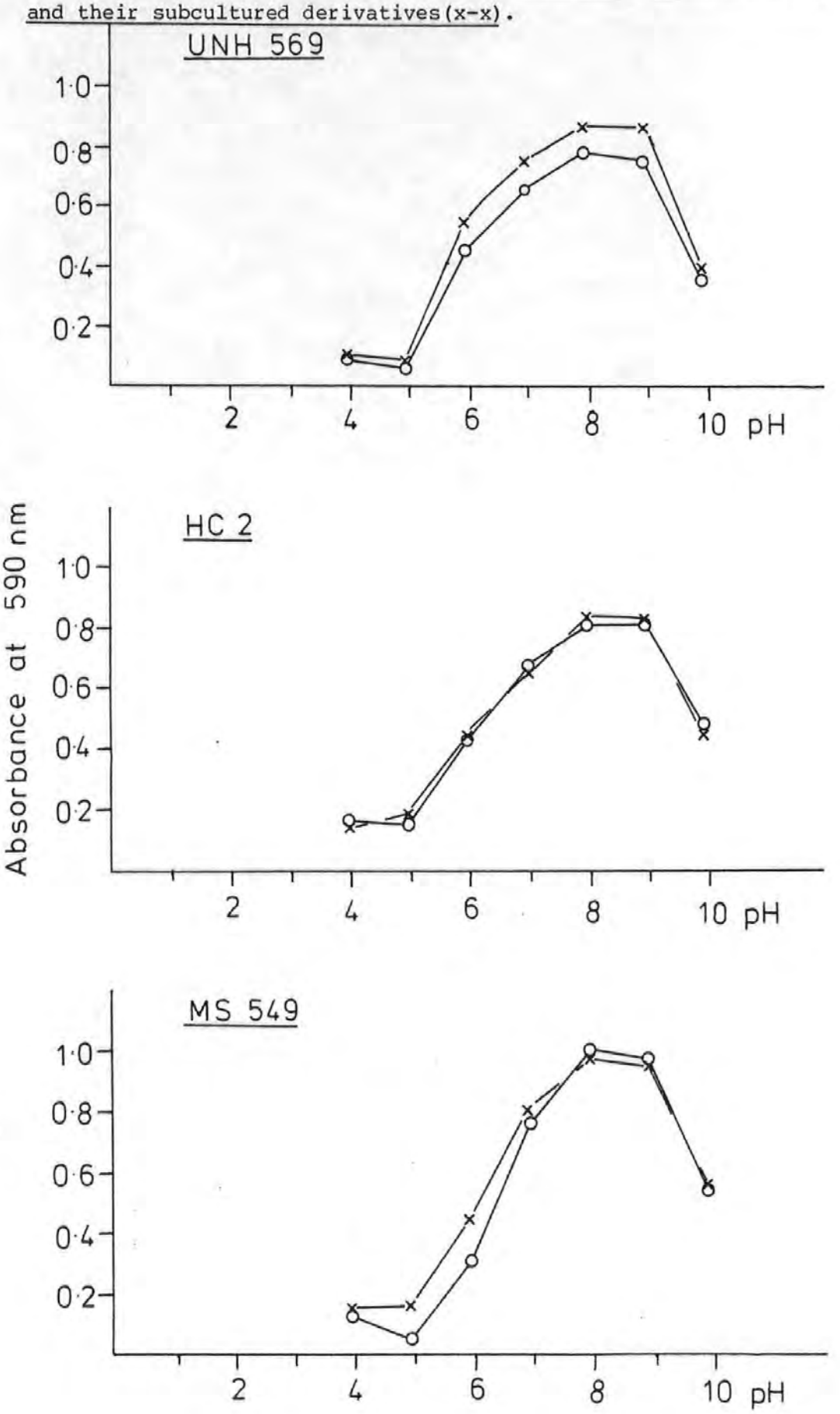
Virulent and low virulence strains were examined for the ability to grow in a range of pH (2.12.8). Absorbance measurements (590 nm) of strains cultured in TSB with a range of hydrogen ion concentration demonstrated strains to have an optimum pH of 8.2 $\pm$ 0.41, active growth taking place between pH 5.5 and 10.5. Statistical analysis carried out on the areas under growth curves revealed virulent strains to have significantly smaller tolerance areas than low virulence strains ($P = 0.05$). Low virulence strains grew better within the experimental pH range (mean area = 60.24 $\pm$ 5.84 cm^2) as compared to virulent strains (Mean area = 45.46 $\pm$ 6.96 cm^2).

Comparisons between parent strains and their subcultured derivatives (Fig. 13) showed subculture to have no significant effect on pH tolerance.

6.2.23 Growth of *V. anguillarum* on selective and differential media as a means of expressing virulence

The potential use of selective and differential media as a means of expressing virulence of *V. anguillarum* strains was assessed (2.12.27). Initial culture on general laboratory media showed most strains to be capable of growing on : Staphylococcal 110, urea, lauryl sulphate, mannitol and tetrathionate media, but not potato

Fig 13 Effect of pH on the growth of 3 strains of V.anguillarum(o-o) and their subcultured derivatives(x-x).



dextrose agar (Table 21).Quantitative comparisons between strains of differing virulence and growth on MacConkey, Czapek Dox and TCBS agars revealed considerable inter-strain variation in growth (Fig. 14) however, no difference between virulence and low virulence could be demonstrated.

Strain UNH 569 and its attenuated and passaged sub-strains cultured on MacConkey agar (Fig. 15), showed that the parent strain and the passaged substrain were able to grow in the presence of bile salts when compared to growth on TSA, whereas the attenuated sub strain exhibited a noticeable reduction in the ability to grow on this medium (Fig. 15).

6.2.24 Results of biochemical identification tests for V. anguillarum and their relationship to virulence

Salient biochemical identification tests for V. anguillarum were carried out on strains of varying virulence (2.12.28) with results generally correlating with established results for this organism (Table 22). Interstrain variations in test results were observed for: growth at 37°C, acid from arabinose and cellobiose, indole production and haemolysis on horse blood agar. Analysis of data with respect to virulence revealed 7 of the 9 virulent strains tested to be indole negative and 9 of the 11 low virulent strains tested to be indole positive. Furthermore, of the 22 strains tested for arabinose utilization, 14 produced acid from this sugar (Table 23).

6.2.25 Relationship between growth rate of V. anguillarum in vitro and virulence

Exponential growth rate was determined for virulent and low virulent strains (2.12.29). Shaken cultures of strains 3022A and

Table 21 Growth of V.anguillarum strains on selective and differential media.

Strain	MacConkey agar	Czapek Dox agar	Staphylococcal 110 medium	Urea agar	Lauryl sulphate agar	Eosin methylene agar	Potato dextrose agar	Mannitol salt agar	Tetrathionate agar	TCBS agar
UNH 569	+++	++	+	++	w[1]	++	–	+	+	++
HC 2	++	++	+	++	++	++	–		++	+
MS 549	++	+	+	++	+	w	–	+	+	–
7/75	+	w	–	+	+	+	–	w	w	w
COB 408	+	w	+	++	+	++	–	+	+	++
1669	+	+	+	++	+	+	–	+	+	w
PB-15	+	+	+	++	+	+	–	+	+	+++

1= weak growth

Fig 14 Bar charts demonstrating the ability of strains to grow on selective and differential media, given as a percentage of growth on TSA.

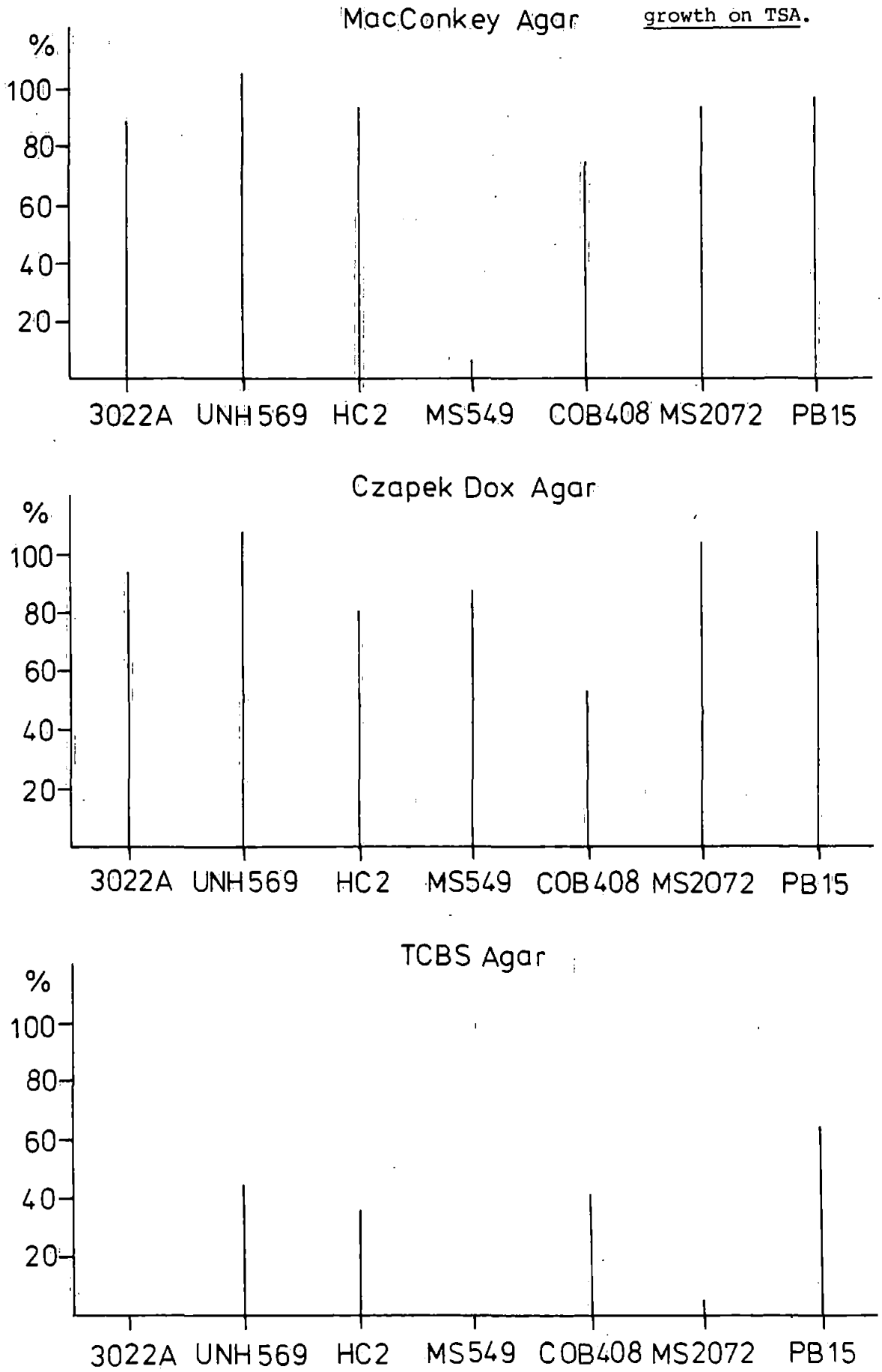


Fig 15 Histogram demonstrating reduced ability of subcultured derivatives of V.anguillarum strain UNH 569 in the presence of bile salts as compared to passaged and parent strains.

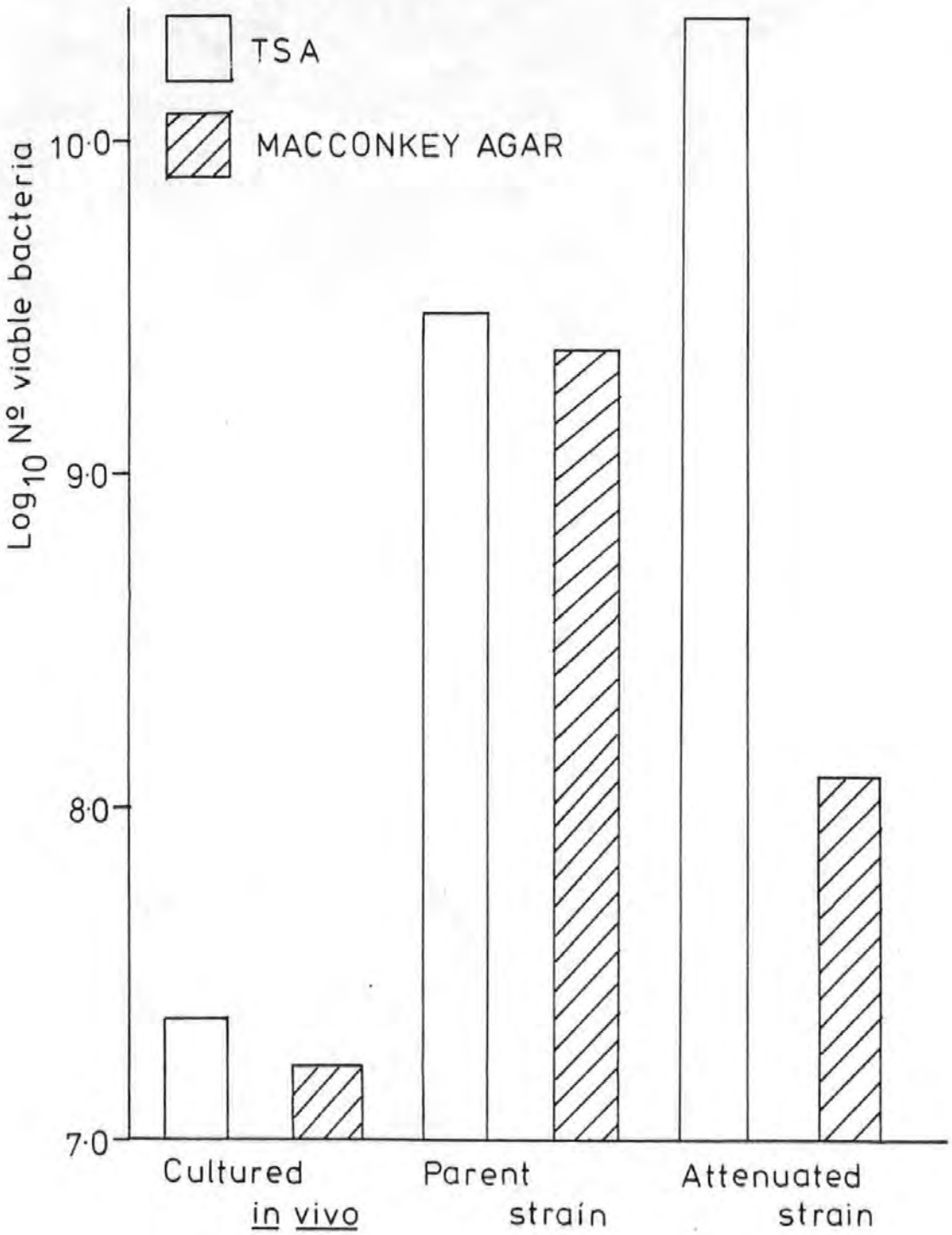


Table 22 General biochemical characteristics of V.anguillarum

Biochemical test	Result
Gram stain	-
Motility	+
Oxidase	+
Catalase	+
Gas from glucose	-
Glucose fermentation	+
Sensitivity to 0/129	+
Sensitivity to methylene blue	+
Growth in TSB + 3% NaCl	+
Growth in TSB + 7% NaCl	-
Growth @ 25°C	+
Growth @ 30°C	+
Growth @ 42°C	-
Acid from carbohydrates	
Dulcitol	+
Fructose	+
Galactose	+
Glucose	+
Lactose	-
Maltose	+
Mannitol	+
Sorbitol	+
Sucrose	+
Gelatin liquefaction	+
Starch hydrolysis	+
H_2S production	-
Urease production	-
Methyl red test	-
Lysine utilization	-
Ornithine utilization	+
Arginine utilization	-

NCMB 1876 = Sorbitol -
7/75 = Dulcitol & Mannitol -
1669 = Starch -
MS 2072 = Sucrose -

Table 23 Biochemical reactions of V.anguillarum and their relation to virulence

Strain	LD 50	Growth @ 37°C	Acid from arabinose	Acid from cellobiose	Indole production	Acetoin production	Haemolysis on horse blood agar
3022A	2.1×10^6_7	+	-	-	-	nd[1]	+
UNH 569	1.1×10^7_7	-	+	-	-	nd	+
HC 2	2.1×10^7_7	+	+	+	-	nd	+
PT-479	4.3×10^7_7	nd	+	-	nd	nd	+
PT-78069	7.6×10^7_8	nd	+	-	nd	nd	+
A1330/78	1.1×10^8_8	nd	+	+	-	nd	+
8.15	1.1×10^8_8	+	-	+	+	+	+
PT-78001	3.3×10^8_8	nd	+	-	-	nd	+
NCMB 829	3.3×10^8_8	-	-	+	+	+	+
MS 549	3.4×10^8_8	+	+	+	-	+	+
775	4.4×10^8_9	nd	+	-	-	nd	+
NCMB 6	$>10^9_9$	-	+	+	+	+	+
NCMB 1873	$>10^9_9$	-	nd	nd	nd	nd	+
NCMB 1876	$>10^9_9$	+	+	+	+	-	+
A20/76	$>10^9_9$	+	+	-	+	+	+
A555/76	$>10^9_9$	+	-	+	+	+	+
1669	$>10^9_9$	+	-	-	-	-	-
COB 408	$>10^9_9$	+	-	+	+	+	+
34/71	$>10^9_9$	+	-	+	+	+	-
7/75	$>10^9_9$	-	+	+	+	-	-
MS 2072	$>10^9_9$	+	-	+	+	+	+
PB-15	$>10^9_9$	+	+	-	+	nd	+
PT 7601	$>10^9$	nd	+	-	-	nd	+

nd= not determined

UNH 569 (virulent) and strains COB 408 and PB-15 (low virulence) were sampled and enumerated (2.2.6) at various time intervals (every 2 hrs for 20 hrs). Triplicate determinations resulted in growth rates (i.e. doubling time) between 0.6 and 0.9 hrs (Table 24). No significant difference in growth rate was observed relating to virulence ($P = 0.01$).

Table 24. Mean generation times for V. anguillarum in relation to virulence

Strain	LD_{50}*	mean generation time†
3022A	2.1×10^6	0.6
UNH 569	1.1×10^7	0.7
COB 408	$>10^9$	0.9
PB-15	$>10^9$	0.85

* LD-50 in eels

† Mean generation time = doubling time (Davis et al., 1980)

6.3.0 DISCUSSION

6.3.1 The pathogenicity of V. anguillarum

The protocol employed for producing vibriosis in eels was similar to that used by other workers in assessing the pathogenicity of V. anguillarum in salmonids (Evelyn, 1971; Traxler & Li, 1972; Schiewe, 1976). Lethal dose 50 values for strains of highest virulence in eels (approximately 10^6 per fish) were considerably higher than the highest value observed in coho salmon (approximately 10^3 viable bacteria (Crosa et al., 1977)) and ayu (reported as 8.75 bacteria,

Jo & Muroga, 1977). Differences in LD 50 values were probably attributable to interspecies variation in host susceptibility to vibriosis.

Virulence determinations of V. anguillarum strains in eels demonstrated interstrain variation in the ability to produce disease; some strains shown to be pathogenic in grey mullet were found to be non-pathogenic to eels. Also, strain 775, shown here to be of low virulence in eels, was found to be highly virulent in salmonids (Crosa et al., 1977). Comparing the results published by Muroga & Egusa (1969) on strain PB-15 it was reported that this strain was highly virulent in eels (A. japonica) while this strain was found to be of low virulence in the freshwater eel (A. anguilla) during the present study. This would suggest that the response of fish to vibriosis can vary even in fish which are of the same genus. This would tend to endorse the theory proposed that V. anguillarum may be host specific (Antipa, 1976; Egidius & Andersen, 1978).

In mammalian research, animals of the same genetic stock are used, reducing variation between animals to a minimum; however, in fish research, workers are obliged to use genetically unrelated stocks. Because of its natural history this is a particular problem with the eel. LD_{50} values should therefore be accompanied by information regarding species, size and age of fish used, and the data interpreted with caution.

Virulence was noticeably enhanced by passage through eels, however subculture did not significantly reduce virulence. Attenuation of V. anguillarum by repeated subculturings was described (Muroga & Egusa, 1969) however, the ability to cause disease was not lost, suggesting that even after several subculturings, the ability to produce disease remains with the strain. The strains that were used

during the present research had all been isolated from fish that had died during disease outbreaks. Reasons why some strains could not cause disease in eels are unclear, but it seems likely that subculture was not responsible for attenuation of strains and it was probable that the strains were primarily incapable of producing vibriosis in eels.

Although there is no clear point dividing virulent from avirulent strains in eels, a comparison between a strain of the highest virulence with a strain of low virulence revealed that the more virulent strain rapidly established itself in the host tissues, while the low virulence strain was rapidly eliminated by the host. This does show that fundamental differences exist between strains which have still to be determined.

6.3.2 Possible links between biochemical properties of V. anguillarum and virulence

The present section was concerned with attempting to link virulence of V. anguillarum in eels with properties exhibited by those strains examined.

From the results (6.2.21) it was evident that antibiotic sensitivity was not linked with virulence. Attenuated substrains of UNH 569 had significantly greater resistance to antibiotics as compared to the passaged strain; the reasons for this were thought to be due to in vivo selection pressures, however similar differences were not exhibited by the other strains tested.

The use of selective and differential media as a means of expressing virulence (6.2.23) failed to reveal any correlation between growth on a given medium and virulence; nevertheless it demonstrated inter-strain variation in the ability to grow on atypical media and

also that V. anguillarum was capable of growing in the presence of bile salts. The apparent reduction in ability to grow in the presence of bile salts exhibited by attenuated substrains would indicate that ability to exist in the gut of fish may be reduced by prolonged culture in vitro.

Biochemical identification tests differentiated strains into V. anguillarum biotype A (forma typica) and Biotype C (forma ophthalmica) after the nomenclature of Nybelin (1935). All strains examined produced acid from mannitol, and so Biotype B was not encountered. There appeared to be a clear correlation between biotype and virulence, with 7 out of 9 virulent strains being type C, while 9 out of 11 low virulence strains were type A. The reasons why virulent strains were generally unable to produce indole are unknown.

Further strain differentiation was achieved on the basis of arabinose utilization. Principal components analysis on V. anguillarum (Håstein & Smith, 1977) revealed two groups:- I (arabinose +ve) and II (arabinose -ve). Examination of the present strains demonstrated a degree of correlation with that of Håstein and Smith (1977). Although details of strain isolation are incomplete, most strains isolated from disease outbreaks in cultured salmonids were generally group II, while isolates from wild fish tended to belong to group I. Statistical analysis was not carried out on the above data, however there appeared to be no strong correlation between virulence and a given group.

6.3.3 Infection mechanisms of V. anguillarum

Most microbial infections start on the mucous membranes of the body surfaces, in the face of host defence mechanisms and commensal bacteria (Smith, 1972). This present section discusses the first attempts to ascertain how V. anguillarum, having entered the host,

overcomes the influences of the host defence system in eels.

Attachment and adherence are important features of an invading pathogen (Smith, 1977). The ability of V. anguillarum to adhere to cells, as indicated by haemagglutination failed to demonstrate agglutination by all strains and no relationship with virulence was demonstrated.

Although there were no differences in the agglutinating ability of V. anguillarum for rabbit and guinea-pig erythrocytes, eel red blood cells were not agglutinated by any of the strains. Haemagglutination studies carried out on A. salmonicida showed variations in the ability to agglutinate red blood cells from different species (Trust et al., 1980). These workers showed that only one strain agglutinated trout and sheep cells while all six strains agglutinated chicken, guinea-pig, rat and human erythrocytes. The binding sites on the erythrocyte membranes are variable and quite different between species (Watt, 1980).

In V. anguillarum only partial inhibition by mannose of haemagglutination was observed, a feature encountered in other members of the genus Vibrio (Tweedy et al., 1968). Inhibition of agglutination by mannose has been reported elsewhere (Duguid et al., 1966; Trust et al., 1980) however, the similarity in haemagglutination inhibition by mannose and fucose observed by Trust and his coworkers was not observed here with V. anguillarum. The reasons for the lack of inhibition by the remaining sugars was probably due to their molecular configuration and the distribution of their hydroxyl groups being incorrect for bacterial attachment. The attachment sites on fish erythrocytes appear not to have been investigated. Inter-species differences between fish blood cells and their unrelatedness to mammalian erythrocytes make the study of bacterial attachment difficult.

Adhesion to mucosal surfaces occurs via hydrophobic interactions, electrostatic forces and van-der Waal's forces (Watt, 1980) which still have to be elucidated for V. anguillarum.

In vitro studies showed that virulent and low virulent strains were phagocytosed by eel splenic macrophages when these cells were provided with fluoresceinated bacteria. It was not clear to what extent phagocytosis was instrumental in the clearance of low virulence strains in vivo. The failure to demonstrate a differential phagocytic response to high and low virulence strains in vitro may be attributable to the fluoresceinating process which could have chemically altered surface properties of the bacteria, which normally operate in protecting against phagocytosis.

The histopathology and bacteriology (Chapter 3) performed in this study have provided evidence that virulent strains of V. anguillarum have means of overcoming the influences of phagocytosis by eel phagocytes. Vibrios were evidently phagocytosed by eel phagocytic cells, as they also were by rainbow trout macrophages (Braun-Nesje et al., 1981); however it was not clear whether ingestion lead to death of the organism. The large numbers of viable bacteria which accumulated in the spleen and kidney tissues during pathogenesis, may have been a consequence of phagocytic activity suggesting that phagocytosed bacteria could remain viable. Mechanisms which this bacterium could employ to prevent digestion have not been investigated and viability studies would have been useful. Intracellular survival has been demonstrated in, for example pathogenic strains of Brucella abortus, Pasteurella pestis and Staphylococcus aureus (Smith, 1976) and Neisseria gonorrhoae (Veale et al., 1977). Virulent strains of Brucella abortus are able to survive and multiply in phagocytes due to a cell wall component which

interferes with the bacteriocidal mechanisms of the phagocytic cells (Smith, 1976).

The V. anguillarum-eel phagocyte relationship requires further study in order to elucidate the interactions. Information on eel phagocytic cells was not encountered and consequently isolated phagocytes were not necessarily given optimum culture conditions.

The present study demonstrated that virulent strains produced coagulase enzymes (6.2.13), which were only produced by one low virulent strain. It has been reported that staphylococci isolated from rabbits had greater resistance to intracellular killing, due to the presence of a surface layer of protein which may result to some extent from the action of free and bound coagulase (Smith, 1976).

6.3.4 Possible toxins and aggressins of V. anguillarum

V. anguillarum was found to lyse all blood types examined. Haemolysin production in vitro was found not to be indicative of virulence, as the ability to lyse horse, eel and plaice red blood cells resided with both virulent and low virulence strains. The noticeable reduction in haemolysin production by attenuated substrains as related to the very haemolytic nature of freshly isolated bacteria would suggest that haemolysin production was enhanced by growth in vivo and may play a role in pathogenesis.

The results suggested the possible presence of two haemolysins produced by some strains of this organism types I and II haemolysis observed here resembling the in vitro haemolysis produced by Staphylococcus aureus (Williams and Harper, 1947). Type I haemolysis appeared similar to the haemolysis produced by α-toxigenic strains of S. aureus, while type II haemolysis was reminiscent of that produced by β-toxigenic strains. These toxins

produced by S. aureus have different effects on the host during pathogenesis (Wiseman, 1975).

Action of the haemolysin appeared to involve the permeability of the erythrocyte cell membrane causing leakage of cytoplasmic haemoglobin while maintaining the overall integrity of the membrane. V. anguillarum was reported to produce lecithinase enzymes (McCarthy et al., 1974) which were thought to be responsible for haemolysis by Clostridium perfringens (Stanier et al., 1972).

Although strains appeared to produce haemolysin at different rates, no correlation could be found relating haemolysin production with virulence. The measurement of haemolysis and relating this to colony size was only a crude means of quantifying production and more accurate techniques should be employed for further studies.

The importance of inflammation in the expression of pathogenic mechanisms by V. anguillarum is unclear. However, the observation of widespread inflammation in the tissues of diseased fish implies that the organism does not produce leucocidins, which have been demonstrated in A. salmonicida (Fuller et al., 1977). Bacterial cell preparations from V. anguillarum did not cause death in eels. Failure to produce death in chinook salmon (Abe, 1972) and coho salmon (Harbell et al., 1979) has been reported; however, Abe reported localised haemorrhaging at the injection site, a feature not observed in eels. Endotoxin would seem to have relatively low toxicity to fish with doses greater than 200 mg Kg^{-1} body wt required to kill fish, as opposed to 0.025 mg Kg^{-1} in calves (Berczi et al., 1966). The rapid host response to endotoxin, characterised by haemosiderin deposits in the liver, suggests that this component may be particularly important in bacterial recognition by the host defence system. Endotoxin injected into mammals has been shown to cause rapid deposition in the liver (Kampschmidt & Schultz,

1961; Kampschmidt & Upchurch, 1961). Abe (1972) found endotoxin to be particularly immunogenic in salmon, giving high agglutination titres. Recognition of V. anguillarum may have been the cause of the inflammatory suppuration observed in tissues.

Crude culture supernatant from V. anguillarum appeared to have no appreciable effect on eels. It remains unclear whether culture conditions were unsuitable for production of lethal factors which were responsible for death in goldfish (Umbriet & Tripp, 1975), or that eels are more resistant to these extracellular secretions.

Failure to detect the toxin by electrophoresis was possibly attributable to its being bound to the erythrocyte membranes and therefore not free in the plasma; or that it was present in undetectable quantities. Related species such as V. cholerae and V. parahaemolyticus both produce toxins which have been shown to bind to membrane glycolipids known as gangliosides (Holmgren, 1978; Takeda et al., 1976). The work of Munn (1980) suggests that the V. anguillarum haemolysin may also bind to erythrocyte membrane gangliosides, which might explain the absence of haemolysin in the infected plasma samples, with available haemolysin bound to the red cells present. It is not clear if the haemolysin produced in vitro is the same component produced during pathogenesis; furthermore, if more than one haemolysin is produced by this organism the relative amounts produced may vary from culture in vivo and in vitro.

The toxin is only produced, in vitro, during the stationary phase of growth (Munn, 1978, 1980), suggesting that this component may also be induced by conditions of low iron. In vivo studies on vibriosis (Chapter 3) demonstrated that host fish respond to infection by increasing the unsaturated iron-binding capacity of the blood and deposit circulatory iron in liver parenchymal cells. It seems likely

that haemolysin may be produced *in vivo* as a consequence of low iron conditions.

The importance of iron in infection is well documented (Bullen *et al.*, 1978; Weinberg, 1978), *in vivo* iron-acquisition being a known virulence mechanism in some bacteria (Rogers, 1973). Although Crosa and his coworkers have demonstrated a plasmid-coded iron-seqestering machanism in *V. anguillarum*, inducible by low iron conditions, the function of haemolysin production is unclear.

Haemolysin production appears to occur *in vivo* during pathogenesis. Red cell lysis in eels receiving low doses of haemolysin preparation, and death caused by higher doses would suggest that this toxin is involved in pathogenesis. The haemosiderin deposition observed in eels injected with haemolysin preparation was thought to be caused by contaminating lipopolysaccharide present in the preparation. Attempts to elucidate the effects of toxin preparations by injection into healthy eels must be interpreted with some caution; the rate of haemolysin production *in vivo* is unknown and in natural infections is probably inflicted upon a weakened eel. Injecting arbitrary amounts of toxin preparation into healthy fish would therefore seem quite different from a natural insult.

Inhibition of pathogenic activity of haemolysin preparations by boiling suggests that this toxin is protein in nature. A thermolabile haemolysin has been demonstrated in *V. parahaemolyticus* (Miwatani *et al.*, 1972) which may be similar to the *V. anguillarum* haemolysin.

Strains shown to be non-haemolytic on blood agar plates were found to be pathogenic to grey mullet; it would have been useful to assess the lytic ability of these strains on mullet blood agar plates. If generally non-haemolytic strains were also non-haemolytic to mullet red cells the role of this enzyme in pathogenesis would be of

doubtful importance. Haemolysis on mullet blood was not determined, as mullet of required size were not available.

Deoxyribonuclease enzymes produced by V. anguillarum may have a possible role in pathogenesis. The apparent nuclear damage observed in diseased tissues may be as a consequence of DNase activity. The inhibitory effect of calcium ions observed during in vitro studies was not investigated and remains obscure; however it was shown that Micrococcus pyogenes var aureus (Staphylococcus aureus) requires calcium to produce a positive DNase reaction in vitro (Weckman & Catlin, 1957). The importance of DNase enzymes in pathogenesis requires further study, it does appear that other fish pathogens including A. salmonicida, A. hydrophila and Ps. aeruginosa produce DNase enzymes (Nord et al., 1975).

Coagulase and lipase activities, although determined in a joint assay system, were clearly differentiated. The significance of lipases in pathogenesis remains obscure. The integrity of lipid droplets observed in diseased hepatic tissues and a lack of reduction in Sudan Black positive material suggests that lipases are not a principal aggressin produced by V. anguillarum. Coagulase enzymes produced by 8 of 11 strains tested may have important implications in the pathogenesis. Coagulase has been shown to be a measure of pathogenicity in Staphylococcus spp (Blair, 1948), and may influence phagocytosis as discussed above.

Although proteases were produced by V. anguillarum, attempts to obtain sufficient quantities for enzyme characterisation were hindered by low yields of these enzymes. Proteases have been demonstrated in A. salmonicida (Sandvik & Hagan, 1968; Shieh & MacLean, 1976) and probably have a major role in pathogenesis of furunculosis (Munro et al., 1980). Proteolytic enzymes have also been

described in other fish pathogens including P. aeruginosa (Nord et al., 1975) and A. hydrophila in which pathogenesis is characterised by lesions produced by proteolytic enzymes (Bullock et al., 1971).

A very distinct reaction was found with elastase production. This is a protease, closely related to trypsin and chymotrypsin. Although this enzyme has been demonstrated in other bacteria pathogenic to fish (Nord et al., 1975) the importance in pathogenesis is unknown.

Amylase produced by all strains of V. anguillarum examined, has been reported in A. hydrophila, but is rarely produced by A. salmonicida, P. fluorescens and P. aeruginosa (Nord et al., 1975). The function of this enzyme in disease is unknown; there is no apparent activity in the tissues during pathogenesis and it is possible that this enzyme is only produced in vitro to assist in starch breakdown of synthetic media.

Certain enzymes not investigated here, have been investigated in closely related species. Esterases have been demonstrated in V. cholerae and V. parahaemolyticus (Adeymo & Colwell, 1967), lecithinases in V. parahaemolyticus (Yanagase, 1968) and mucinases in V. cholerae (Burnet, 1948). It would have been of particular interest to examine the presence of the above enzymes in V. anguillarum, in particular mucinases which are responsible for the desquamation of guinea-pig epithelia (Burnet, 1948).

Of the enzymes examined it was apparent that the more virulent strains were able to produce all the enzymes tested for, while low virulence strains exhibited only limited enzyme production. DNase produced by virulent strains was depressed by the presence of calcium ions, a feature not shared by low virulence strains. The reasons for this are unknown. Subculture of strains failed to show any major

differences in enzyme production.

The electronlucent zone observed around V. anguillarum in infected tissues by electron microscopy closely resembled the zone observed by Draper & D'Arcy Hart (1975), and Marchiondo et al. (1980) around Mycobacterium lepraemurium in armadillos. Capsular analysis confirmed that the zone around V. anguillarum was not a capsule but was caused by the action of localised lytic enzymes; in contrast, the zones observed in leprotic tissues have been shown to be composed of lipid of bacterial origin. Furthermore, if the zone around V. anguillarum had been of a lipid nature it would have been stained black by the treatment of osmium tetroxide during preparation for electron microscopy.

6.3.5 Colonial morphology and ultrastructure of V. anguillarum

Colonial morphology of V. anguillarum did not correlate with virulence in eels. Colonial morphology has been shown to be indicative of virulence in A. salomonicida with rough colonies being of lower virulence than smooth colonial types (Anderson, 1972), due to the possession of an additional protein layer (Udey & Fryer, 1978). From colonial observations on gonococci it has been demonstrated that a relationship exists between colonial morphology and virulence such that, for example, opaque colonies are killed in serum while transparent colonies are able to survive serum killing factors (James & Swanson, 1978).

The change in colony appearance between V. anguillarum strain UNH 569 and the subcultured derivative could not be linked directly to virulence, as subculture did not significantly reduce virulence. Nevertheless, when attenuated substrains were injected into fish, the isolated bacteria were of the shiny domed colonial type, indicating

that colonial morphology is influenced by culture *in vivo*.

From ultrastructural observations it was evident that ammonium molybdate produced better results than phosphotungstic acid, with the latter causing loss of detail of surface structure. Gold-palladium was the superior shadowing agent producing a fine grained background, as opposed to gold alone which produced a granular background making observation of fine detail difficult.

The ultrastructure of this species has not been reported previously; however, the present studies demonstrated a typical Gram negative membrane topography. The rugose outer membrane observed in all strains examined by negative staining, ultrathin sectioning and SEM, appeared to be a common feature of *V. anguillarum*. The g2 layer of *E. coli* described as peptidoglycan (De Petris, 1967) (Fig. 12) was present in *V. anguillarum*. The removal of this layer by treatment with EDTA and lysozyme (2.12.17) suggested that the layer correlating with g2 in *E. coli*, is also peptidoglycan. In *E. coli*, the cytoplasmic membrane was described as having 3 layers (De Petris, 1967); in *V. anguillarum*, only a single layer was resolved.

Dimensions of *V. anguillarum* are similar to those of other members of the genus *Vibrio*. Differences in size between negatively stained and metal-shadowed preparations have been observed previously in *Vibrio* spp by Tweedy *et al.* (1968); as with the present study they found that metal-shadowed preparations were slightly larger than negatively stained bacteria. It was thought that size differences in *V. anguillarum* and other species were attributable to the shadowing procedure causing the bacterial cell to become flattened. This would explain the abberation of the typically wrinkled membrane and the highlighting of the cytoplasmic inclusions. The nature of the cytoplasmic inclusions remains unclear, but appear to be similar to

those observed in Vibrio fetus (Campylobacter fetus) by Ritchie et al (1966).

A possible virulence factor was revealed by electron microscopy with multiflagellate bacteria only being observed in virulent strains, a feature not lost by attenuation. Motility of V. cholerae has been reported as being essential for the penetration of mucosal membranes of rabbit brush border cells (Jones et al., 1976); which, considering the invasive nature of V. anguillarum, could be of importance in pathogenesis. However, monoflagellate strains of low virulence in eels could produce disease in grey mullet, thus shedding doubt on the importance of multiflagellation in pathogenesis.

In addition to conferring motility, flagella have been reported to have adhesive properties. Jones et al. (1976) demonstrated a correlation between loss of motility and loss of adhesive factors in V. cholerae. Comparisons made between various species of the genus Vibrio have shown flagella antigenicity to be similar within species tested, including V. anguillarum (Shinoda et al., 1976). Antigenic similarity between V. cholerae and V. anguillarum may indicate similar adhesion properties present in both species. In flagella adhesion, it was postulated that the flagella sheath was the true adhesion (Watt, 1980). Although multiflagellate strains were observed in the present studies, their relation to adherence and penetration requires further research.

V. anguillarum has been previously described as having only single flagella (Håstein, 1974; Roberts, 1978), however the present study is the first to report that multiflagellate strains do occur. Structurally, the V. anguillarum flagella appear to be similar to those described for V. metchnikovi (Glauert et al., 1963; Follet & Gordon, 1963; Ogasawara & Kuno, 1964). In V. metchnikovi the flagella were

found to have a central core of flagellin surrounded by a sheath of outermembrane-like material. The biochemical properties of the V. anguillarum flagellum have still to be examined.

The terminal swelling observed in V. anguillarum flagella appears similar but slightly smaller than that observed in V. cholerae (biotype el tor) by Rowles et al. (1976). They suggested that this terminal swelling may be composed of either cell wall material carried away as the new flagella emerge from the cell, or assemblies of less well organised flagellin subunits.

Although some strains of V. anguillarum were shown to have pili, this feature was only evident in relatively few cells. Pili have been implicated as important mechanisms of attachment (Watt, 1980). The work of Duguid & Gillies (1958) emphasises the importance of critical culture conditions necessary for the production of pili, which were not fully investigated here for V. anguillarum. Further reasons for the lack of pilated individuals could be due to selection pressures, by growth in vitro, against this feature. For example in N. gonorrhoeae, pili are invariably present in freshly isolated bacteria from human patients however, subculture selects against pilated forms (Jephcott et al., 1971).

The importance of adherence in the invasion of the fish host by V. anguillarum requires further investigation, however, from studies of other pathogenic bacteria, pili are not the only prerequisite for cellular adherence.

Haemagglutination has been used as a means of detecting bacterial pili and the ability to adhere to cells (Duguid & Gillies, 1957; Duguid & Gillies, 1958; Duguid et al., 1966), although pilation did not appear to be exclusively responsible for adherence in gonococci (Tebbut et al., 1976) and salmonellae (Duguid et al., 1966).

6.3.6 Envelope proteins and plasmids of V. anguillarum

The methodology employed for the analysis of V. anguillarum cell envelopes, provided protein preparations producing high resolution of polypeptides on polyacrylamide gels in the presence of SDS. Chemical extraction using Triton X-100 produced similar protein profiles to those resulting from the separation of sphaeroplast preparations, and seems to have been particularly useful for the analysis of other Gram negative membranes (Schnaitman, 1971a & 1971b). Extraction by guanidine hydrochloride appeared to cause leakage of cytoplasmic proteins which although useful in analysing for intra-strain protein variation for whole cell proteins, had relatively little use in studies focussed on the cell envelope proteins only. Lithium acetate extracted relatively fewer proteins than the other techniques, but still facilitated the demonstration of major proteins. The preparation resulting from EDTA extraction proved too deliquescent for accurate sample preparation and was consequently not examined by electrophoresis.

Analysis of sphaeroplasts was thought to be the most suitable means of examining envelope proteins. Separation of cell envelopes from other bacteria components provided a means of focussing solely on the cell envelope proteins; furthermore chemical extraction has been shown to denature proteins during extraction particularly when denaturation involves solubilisation of hydrophobic components in aqueous solvents (Maddy & Dunn, 1976). Formation of these membrane components in aqueous solvents produces proteins which are different from the original membrane proteins. Although chemical analysis still has potential in membrane protein analysis, as particular features can become pronounced as a consequence of denaturation.

Knowledge of the relationship between virulence in V. anguillarum and membrane proteins have facilitated better understanding of

virulence mechanisms in salmonids as shown by Crosa and his coworkers, recently. The virulent strain 775, was shown to contain an outer membrane protein of 86000 daltons, induced by low iron conditions (Crosa & Hodges, 1981) which is probably responsible for the association between possession of a large plasmid and virulence (see below). The effects of environmental factors on the outer membrane profile were not studied here, but it is likely that other membrane - associated virulence factors are inducible.

The presence of a major protein located in the outermembrane was revealed, ranging from 28000 - 36000 daltons depending on sample preparation. Studies on E. coli have shown this bacterium to have a major protein in its outermembrane of 36500 daltons (Boyd & Holland, 1979), considered to be matrix protein. The term matrix protein was originally applied to outer membrane protein (omp) F of E. coli (Rosenbusch, 1974) and more recently expanded to refer to constitutively expressed pore-forming proteins of E. coli and S. typhimurium omp (Osborn & Wu, 1980). In E. coli, this pore-forming protein or porin has been shown to allow selective passage of small hydrophilic molecules such as glucose, sucrose and amino acids with the exclusion of larger molecular weight compounds such as bile (Nakae, 1976; Boyd & Holland, 1980). An absence of the porin, decreased affinity for most nutrients (Bavoil & Nikaido, 1977). It seems possible that the major outer membrane protein of V. anguillarum is also a pore protein which could play a role in selective uptake of nutrients in the presence of bile, as would be experienced in the gut of fish.

In a recent publication (Buckley et al., 1981) the envelope proteins of V. anguillarum biotype 1 (type A by Nybelin, 1935) were examined. Electrophoretic separation of proteins from envelopes

prepared by passage through a French pressure cell, revealed a correlation between protein profiles and geographical location. Applying the same analysis to the present data it was found that biotype 1 strains PB-15 (Japan), MS 2072 (USA) and COB 408 (France) had noticeably different protein profiles (Plate 43, tracks 1-3). This would tend to endorse the theories of Buckley et al. (1981) that within V. anguillarum biotype 1, strains of similar geographical location may be of clonal decent. When the association was extended to encompass all strains, regardless of biotype, the relationship between profile and geographic location became less distinct. Evidently, a more comprehensive examination of strains from different biotypes and geographical locations is required.

Buckley et al. (1981) described a heat modifiable envelope protein of 33000 daltons and this would be an interesting topic for future research, as heat-modifiable proteins have been shown to be important in the virulence of other Gram negative organisms, with roles in adhesion to epithelial cells in leucocytic-associated reactions and in resistance to bacteriocidal activity of serum (Buckley et al., 1981).

Studies on the membrane proteins of V. anguillarum strain 775 by Crosa and his coworkers demonstrated an outermembrane protein of 86000 daltons which was induced by low iron conditions. This protein was not encountered during the present study but it does illustrate that some virulence factors of this organism are induced. To what extent other virulence factors are present which are only produced under specific environmental conditions remains to be investigated.

The possibility of quantitative protein differences being involved in V. anguillarum pathogenicity, prompted the use of crossed immuno electrophoresis. For example, examination of Shigella flexneri

envelope proteins revealed quantitative differences in the amounts of protein present relating to virulence in guinea-pigs, as opposed to qualitative protein variation (Witkowska, Adamus, Mulczk & Romanowska, 1980). Although only preliminary studies were carried out, CIE was adapted to use with V. anguillarum (2.12.23) providing a useful tool for future research. The studies carried out revealed apparent quantitative differences between strains (Plate 48) but these differences were not assessed with respect to virulence.

The size of plasmids isolated from strains of V. anguillarum were in accordance with those of Crosa et al. (1977). Although some of the more virulent strains possessed the 47 megadalton plasmid, several strains did not. In addition, many of the strains of low virulence to eels possessed the plasmid. Crosa et al. (1977) examined 12 strains of V. anguillarum and concluded that virulence for coho salmon was closely associated with possession of the large plasmid. Recently, the plasmid, characteristic of high virulence strains, has been shown to code for a highly efficient iron-sequestering system which probably facilitates colonization by the pathogen in spite of iron-witholding mechanisms by the host (Crosa, 1980; Crosa & Hodges, 1981). The results of Schiewe (1976) show that one strain of V. anguillarum although lacking the large plasmid was highly virulent to salmon. Clearly, presence or absence of the plasmid does not alone determine the pathogenicity, but in some strains plasmid possession may enhance the capacity to produce disease.

The observed partial correlation between colistin or polymyxin resistance and plasmid possession has not been previously described. Polymyxin antibiotics are known to bind to the cell membrane and disrupt its function, and bacterial resistance is probably due to altered cell envelopes (Gilleland & Murray, 1976). The relationship

between membrane proteins and resistance to polymyxin could be determined by comparing profiles of parent strains with these same strains having been cured of the plasmid.

The present chapter has been concerned with elucidating the pathogenic mechanisms and possible virulence factors of V. anguillarum. Virulent strains multiplied within the fish tissues and caused disease, while low virulence strains were unable to multiply and were rapidly eradicated. It appears likely that a major virulence factor is concerned in avoiding host defence mechanisms or in obtaining essential nutritional requirements. Although a clear link between toxin production and virulence was not observed, extracellular products may play a role in pathogenesis with pathogenic mechanisms being host specific.

The study of this organism would be greatly enhanced by a better knowledge of induced virulence factors. In addition, tissue culture techniques would prove useful for the study of attachment and cellular penetration.

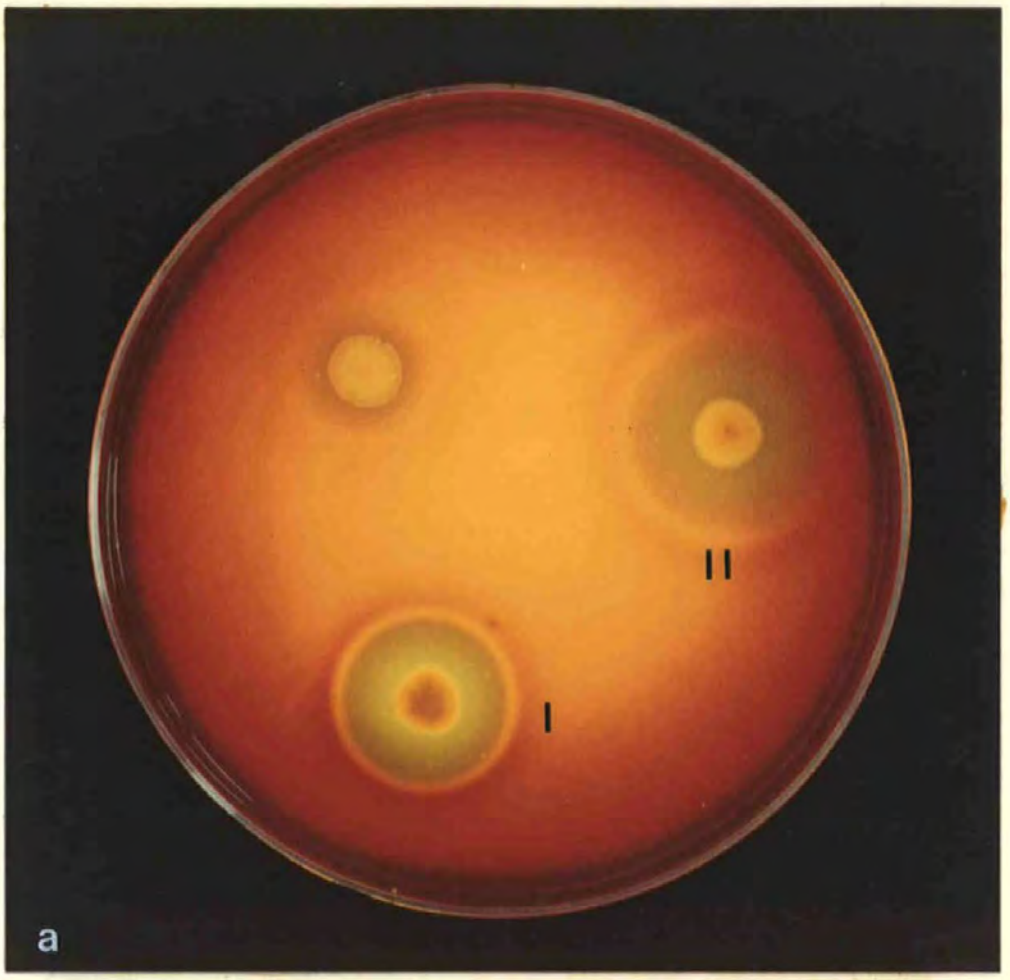
From the analysis of these findings it was deduced that the fish species used is difficult to infect and that natural infections in eels is a complex, yet illdefined phenomenon.

Plate 27 a) Types of haemolysis exhibited by V.anguillarum on horse blood agar:- Type I

Type II

Weak haemolysis.

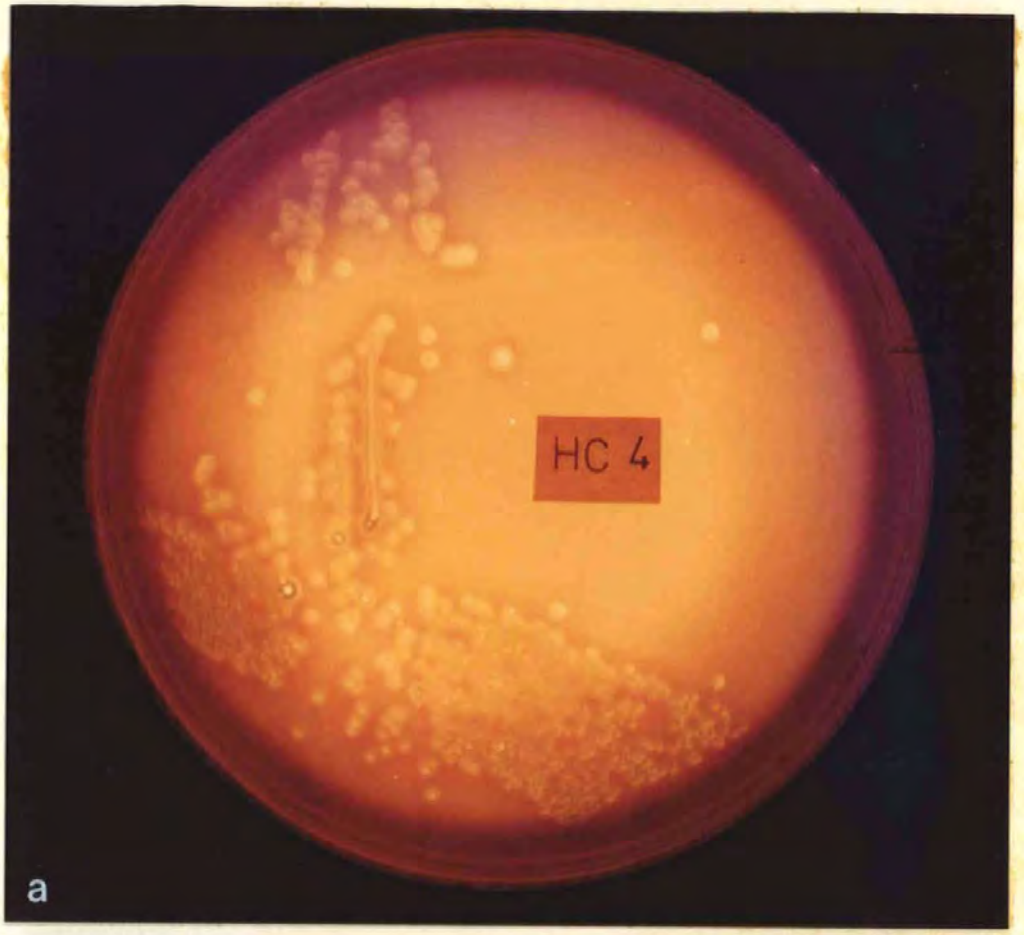
Plate 27 b) Electron micrograph of erythrocyte 'ghost' from the lytic zone around V.anguillarum cultured on horse blood agar(x 8000,bar=1.25µm).


II
I
a


cyt
b

Plate 28a) Haemolysis of horse erythrocytes by V.anguillarum strain UNH 569 (HC 4) cultured on blood agar.

Plate 28b) Haemolysis of horse erythrocytes by V.anguillarum strain HC 2 cultured on blood agar.


HC 4
a

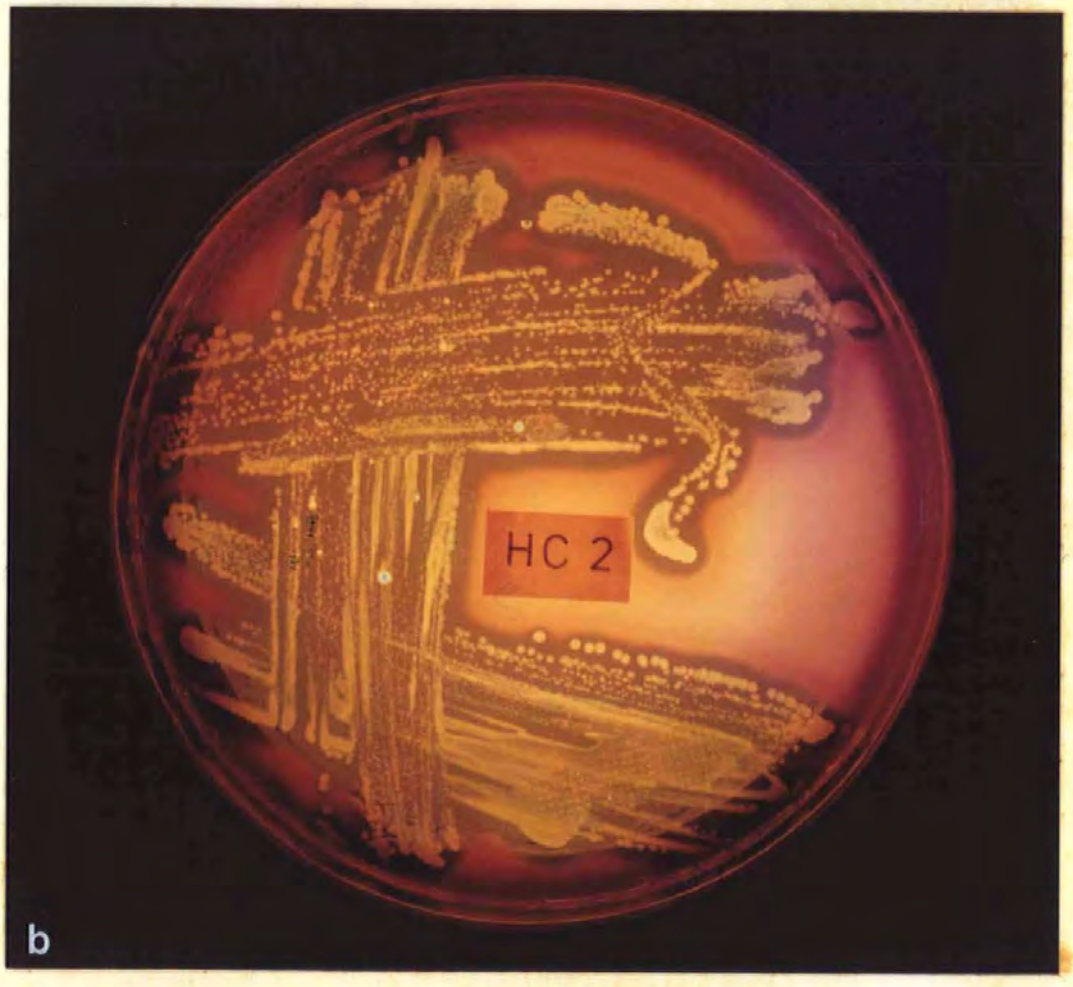
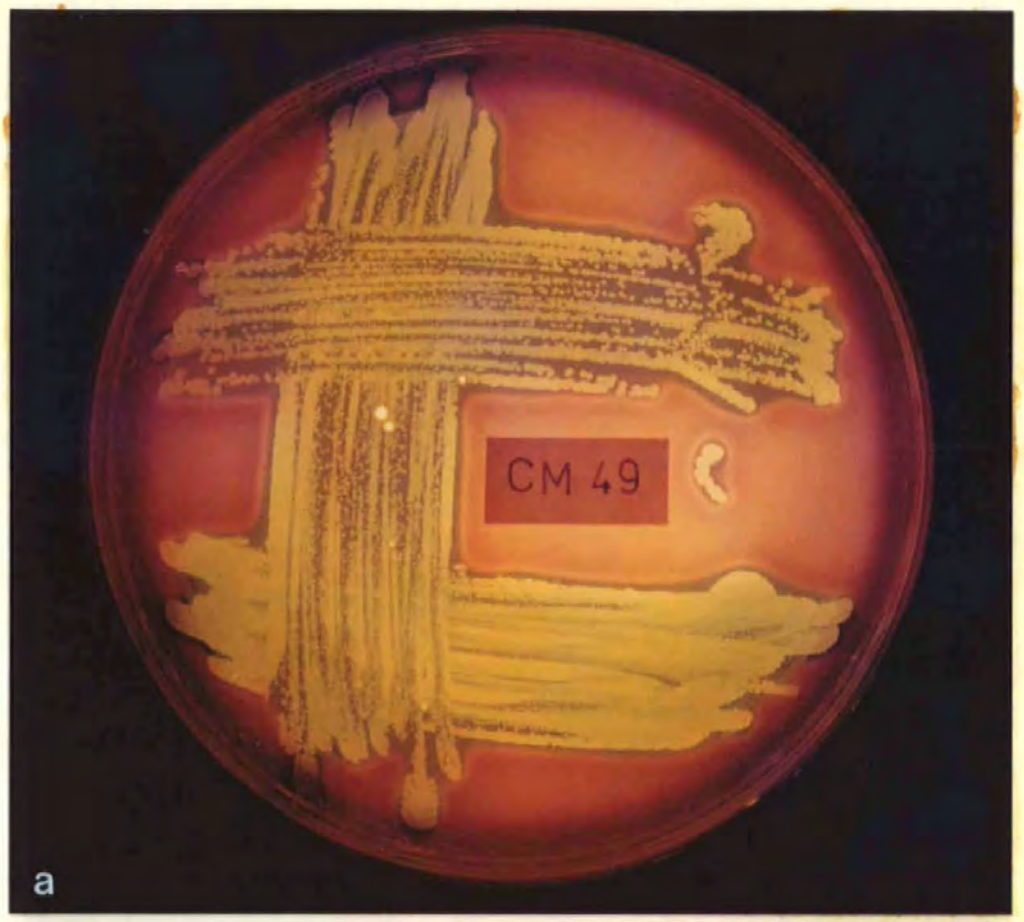
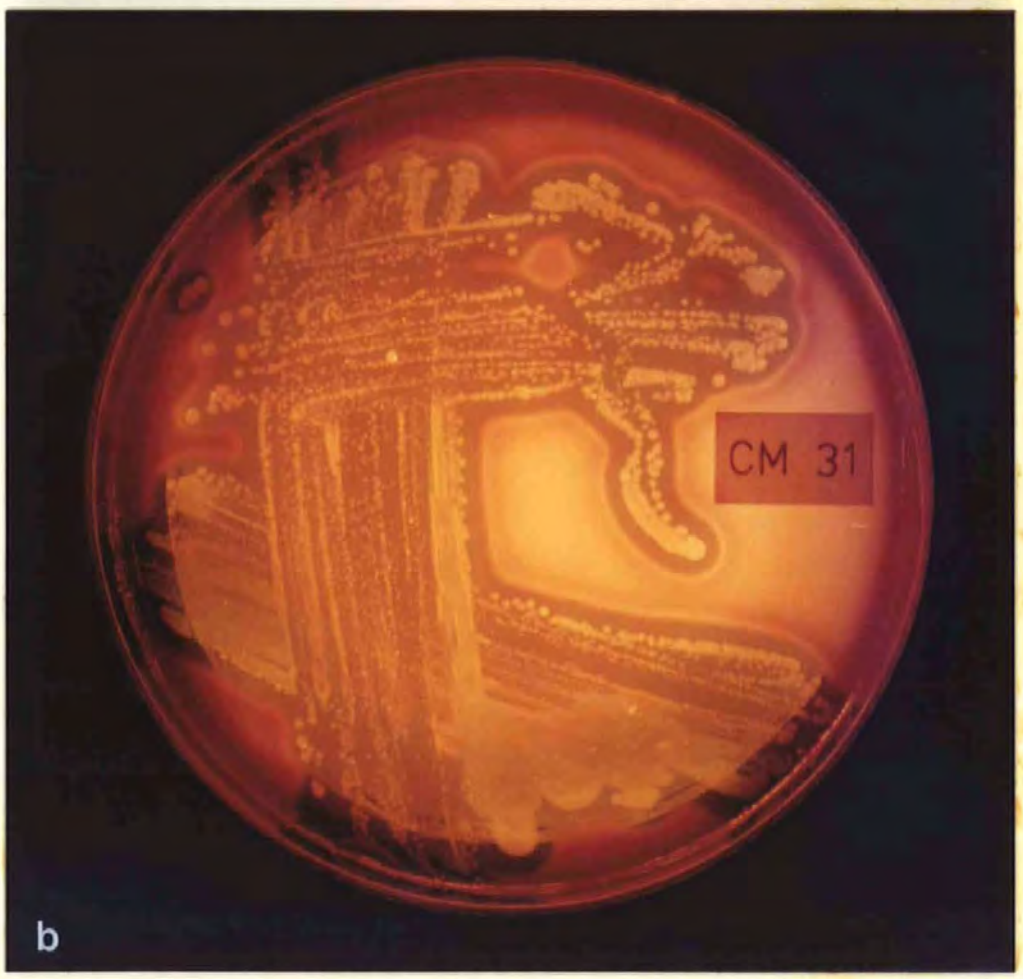

HC 2
b

Plate 29a) Haemolysis of horse erythrocytes by V.anguillarum strain MS 549 (CM 49) cultured on blood agar.

Plate 29b) Haemolysis of horse erythrocytes by V.anguillarum strain COB 408 (CM 31) cultured on blood agar.



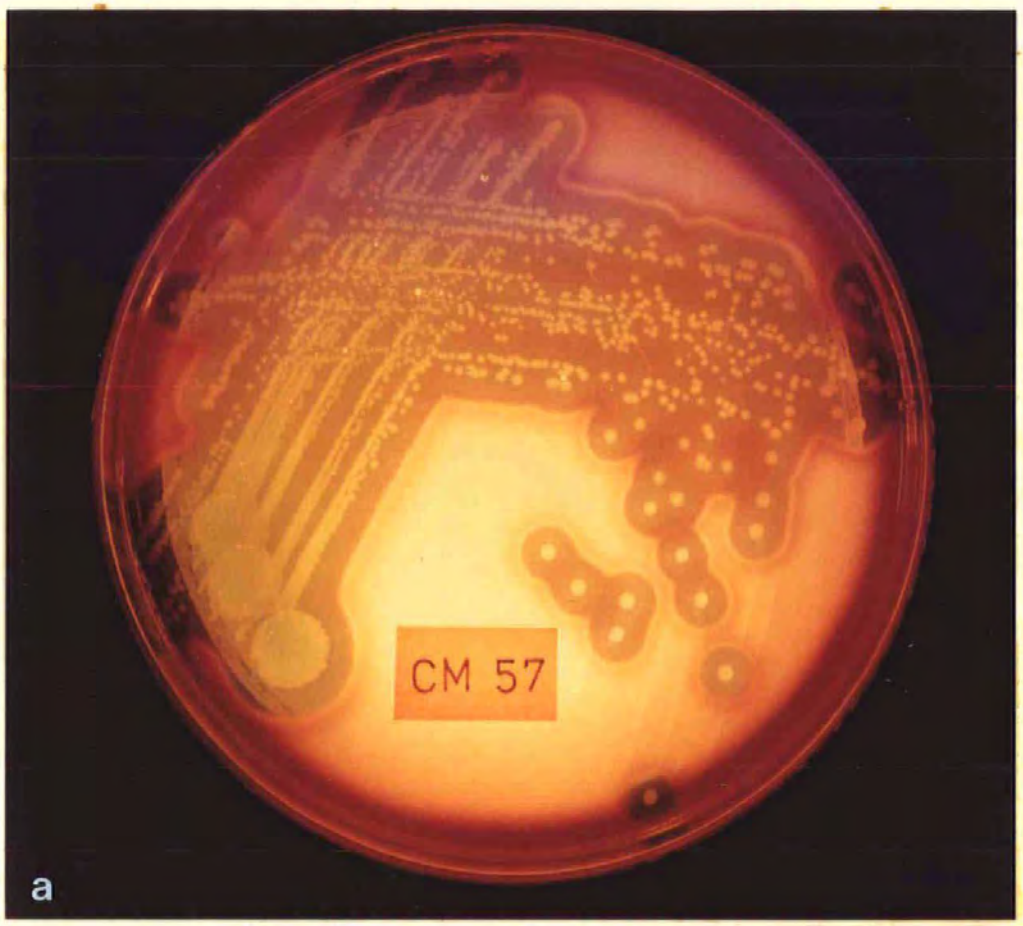
a



b

Plate 30a) Haemolysis of horse erythrocytes by V.anguillarum strain PB-15 (CM 57) cultured on blood agar.

Plate 30a) Haemolysis of horse erythrocytes by V.anguillarum strain 7/75 (CM 16) cultured on blood agar.


CM 57
a

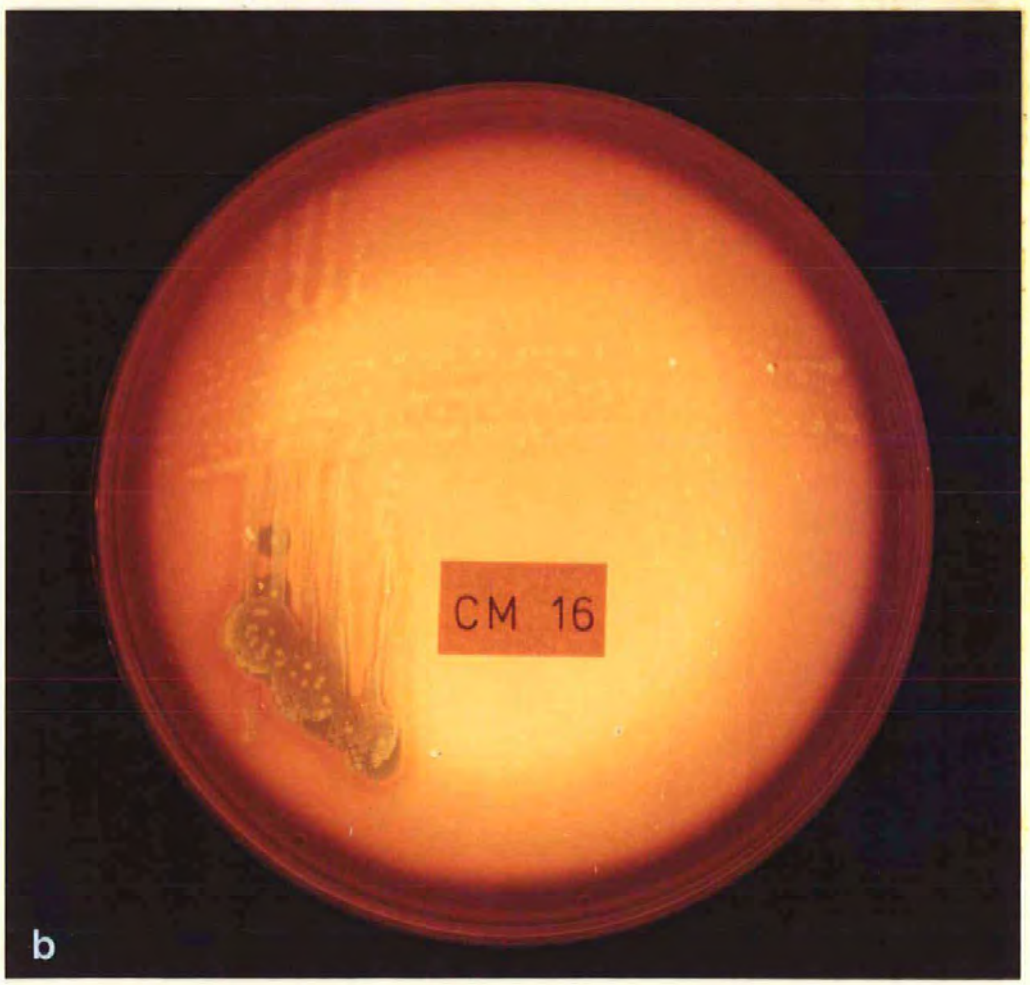
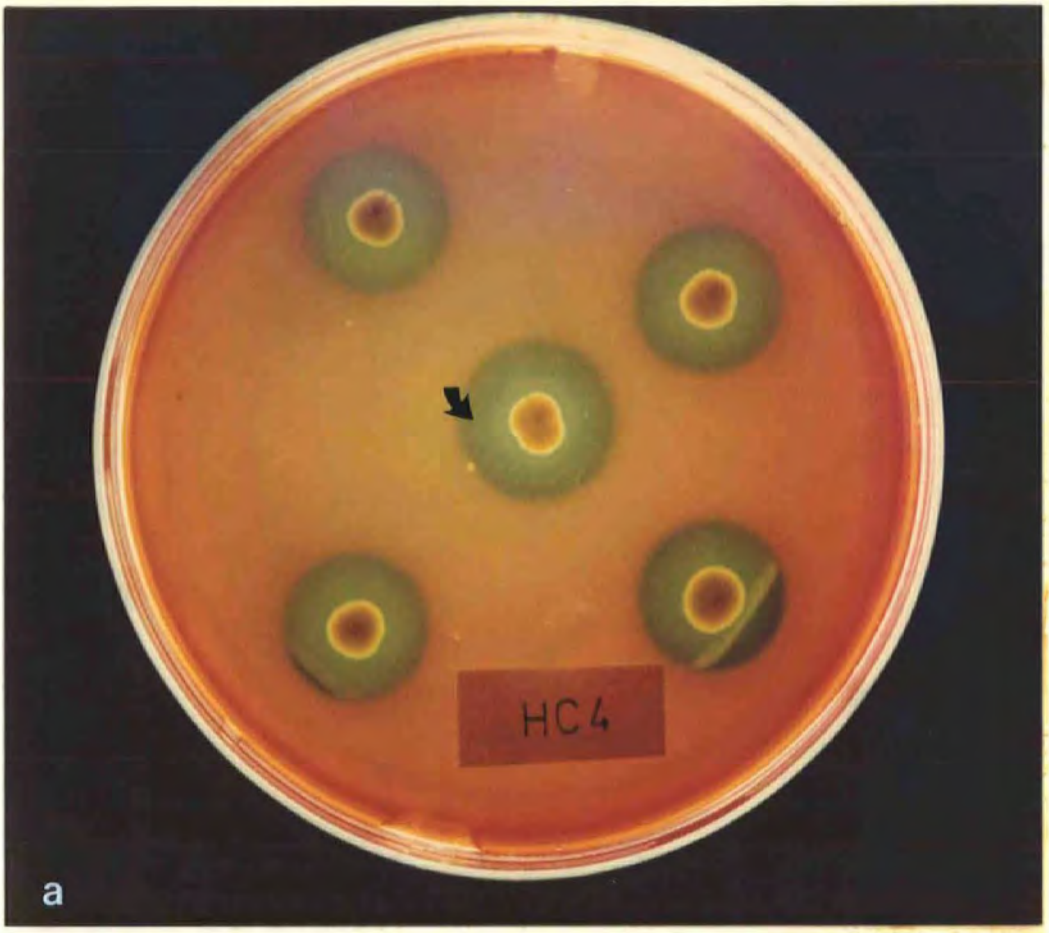

CM 16
b

Plate 31a) Haemolysis zones (arrowed) produced by V.anguillarum strain UNH 569 (HC 4) 'parent strain'.

Plate 31b) Haemolysis zones(arrowed) produced by V.anguillarum strain UNH 569 (HC 4) subcultured derivative.


HC4
a


HC4 attd x 20
b

Plate 32 Light micrograph showing histochemical staining of infected eel spleen(alcian blue, x 94 ,bar=100μm).

e=erythrocytes

s=splenic parenchymal cells

arrowed=melanomacrophage centres.

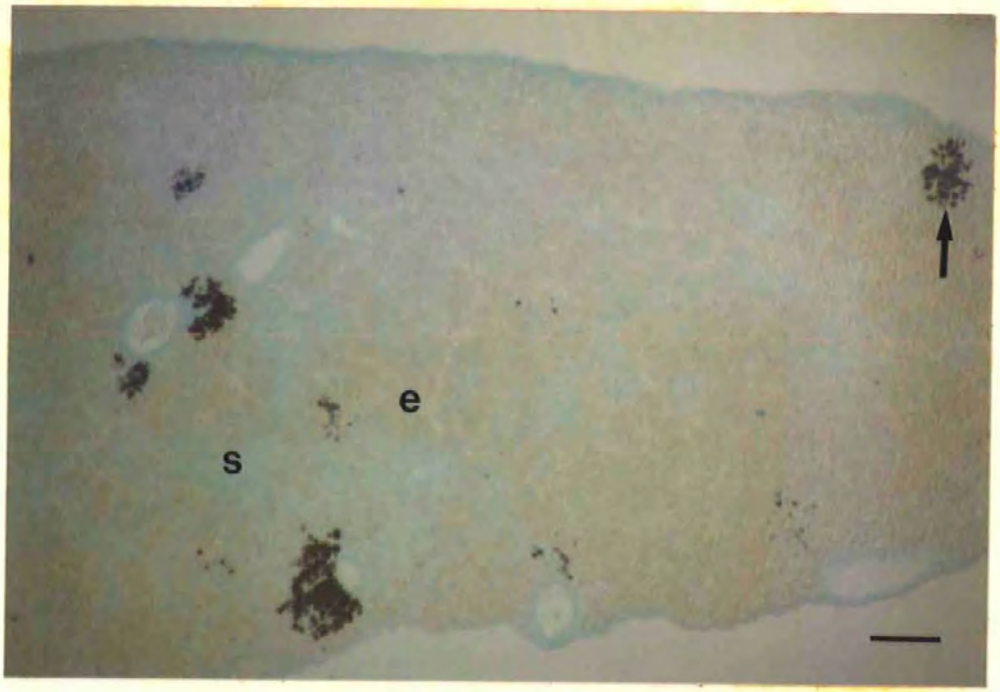

e
s

Plate 33a) Electron micrograph of 'vibrio-like' bacteria(B) in infected eel liver demonstrating electron-translucent zone(tz) around bacteria (x 11000,bar=1μm).

Plate 33b) Electron micrograph of 'vibrio-like' bacteria(B) in infected eel spleen demonstrating electron-translucent zone (tz) around bacteria (x 68000,bar=0.25μm).

Plate 33c) Electron micrograph of 'vibrio-like' bacteria in infected eel heart demonstrating zone (◆▶) between bacteria(B) and host tissue(ht) (x 19000,bar=0.5μm).

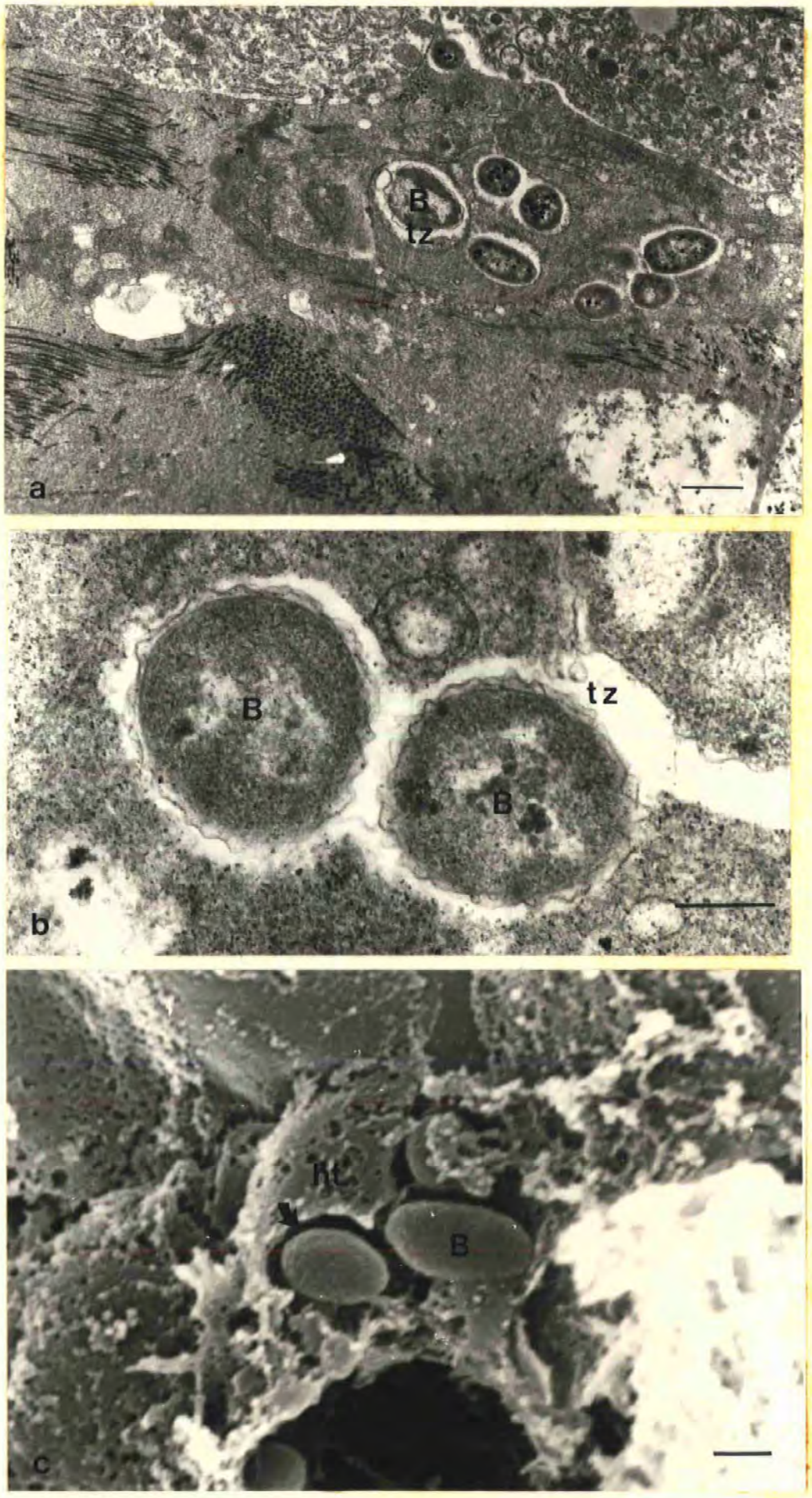
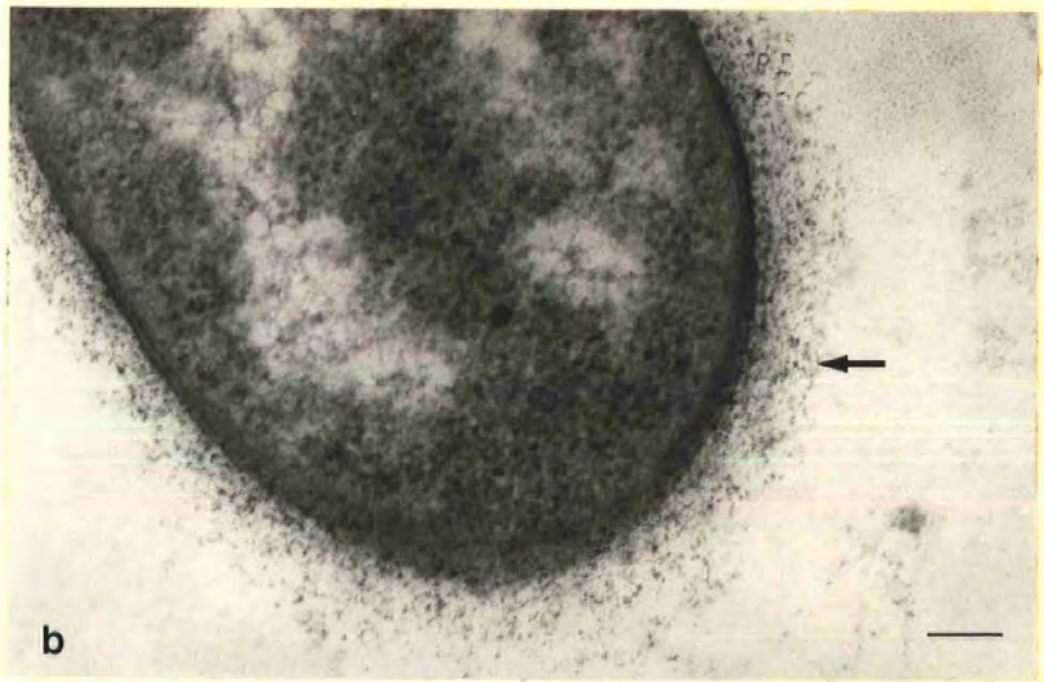

B
tz
a
B
tz
B
b
hc
B
c

Plate 34 a) Electron micrograph of agar cultured V.anguillarum stained with ruthenium red,showing absence of capsular material (x 97000,bar=0.25μm).

Plate 34 b) Electron micrograph of broth cultured Klebsiella pneumoniae stained with ruthenium red showing a positive capsule reaction(arrowed) (x 100 000,bar=0.1μm).

Plate 34 c) Electron micrograph of agar cultured Klebsiella pneumoniae showing capsular material (arrowed) in the absence of ruthenium red (x 64000,bar=0.25μm).


a


b

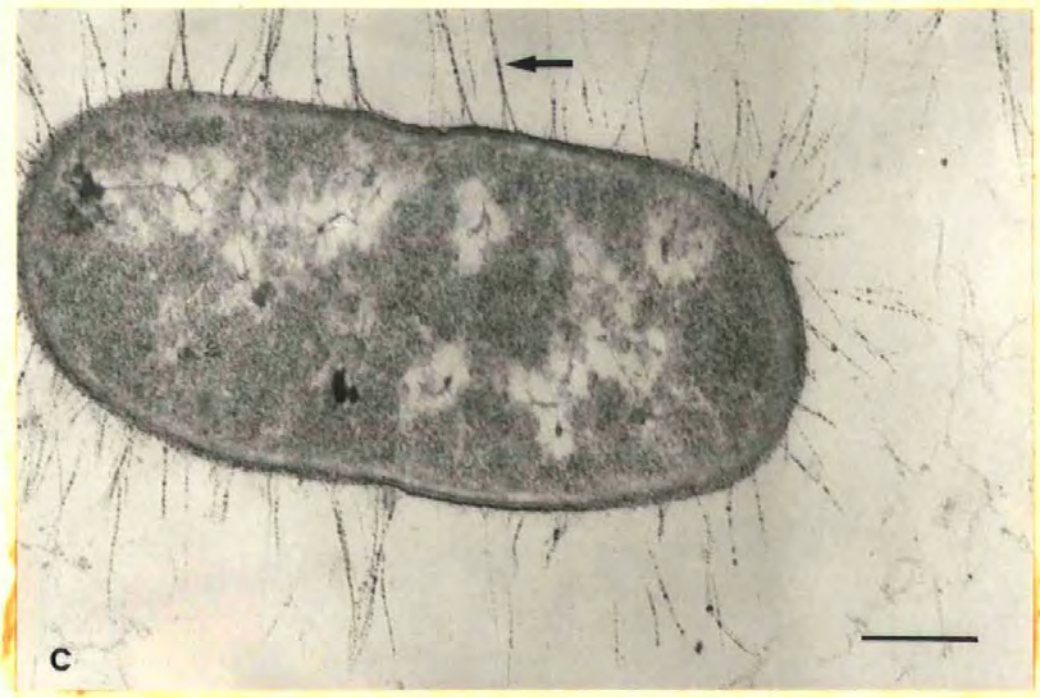

c

Plate 35a) Culture of V.anguillarum strain UNH 569 on horse blood agar showing 'domed' colonial morphology.

Plate 35b) Culture of V.anguillarum strain UNH 569, subcultured derivative, on horse blood agar showing 'umbonate' colonial morphology.


a


b

Plate 36 Electron micrograph of negative stained V.anguillarum strain UNH 569 of long form with :-

a) 1 flagellum (x 42000,bar=0.5µm).

b) 2 flagella (x 42000,bar=0.5µm).

c) 3 flagella (x 26750,bar=1µm).

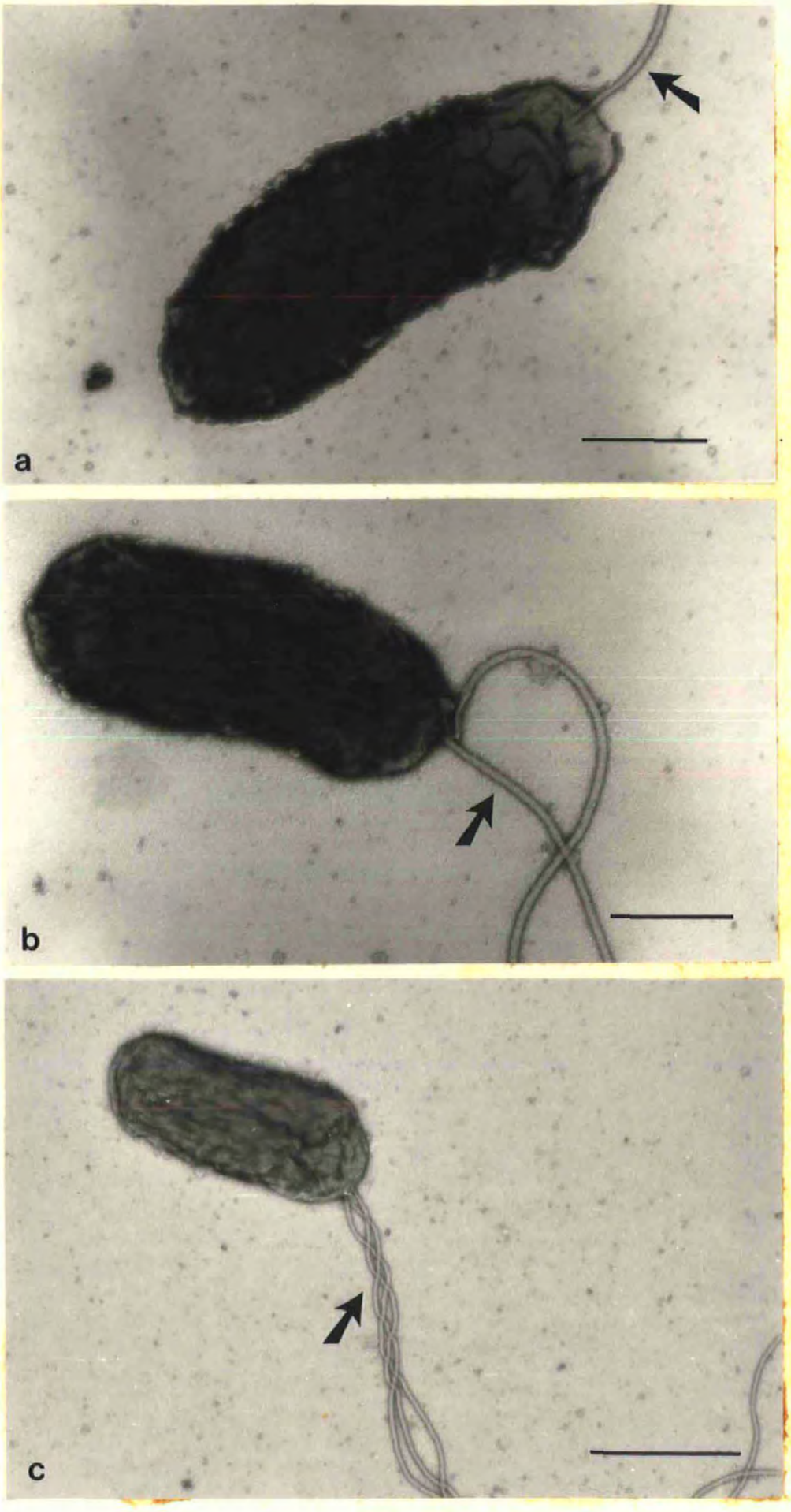

a
b
c

Plate 37 a) Electron micrograph of negative stained V.anguillarum strain UNH 569 of short form with 2 flagella(x 40000,bar=0.5µm).

Plate 37 b) Electron micrograph of negative stained V.anguillarum strain UNH 569 of short form with 3 flagella(x 30000,bar= 0.5µm).


a

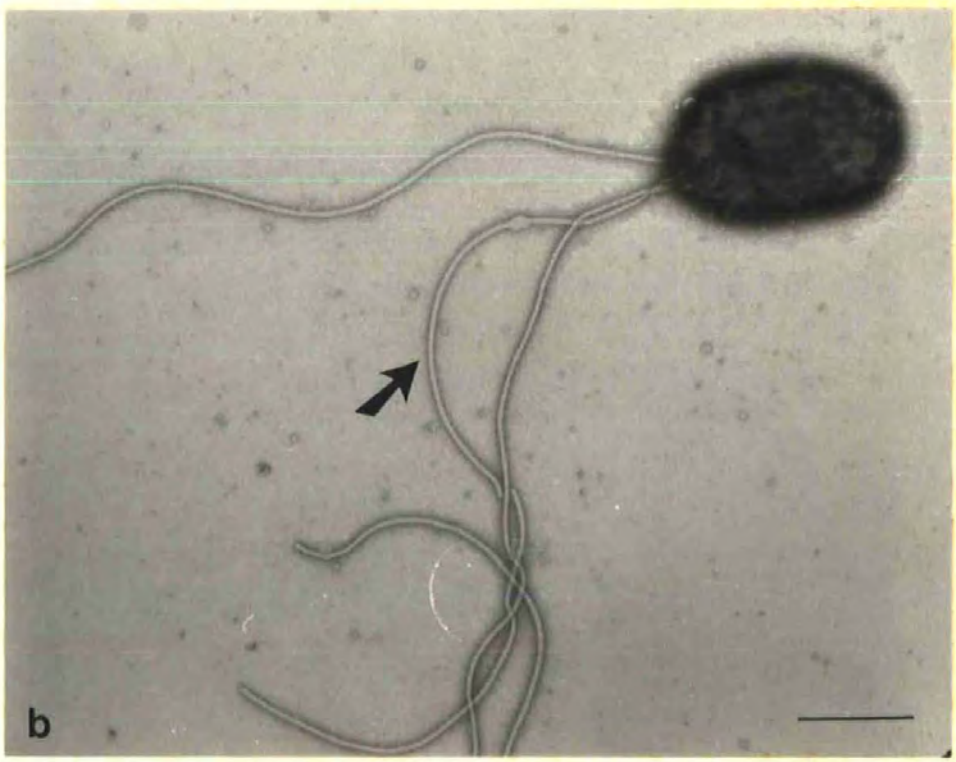
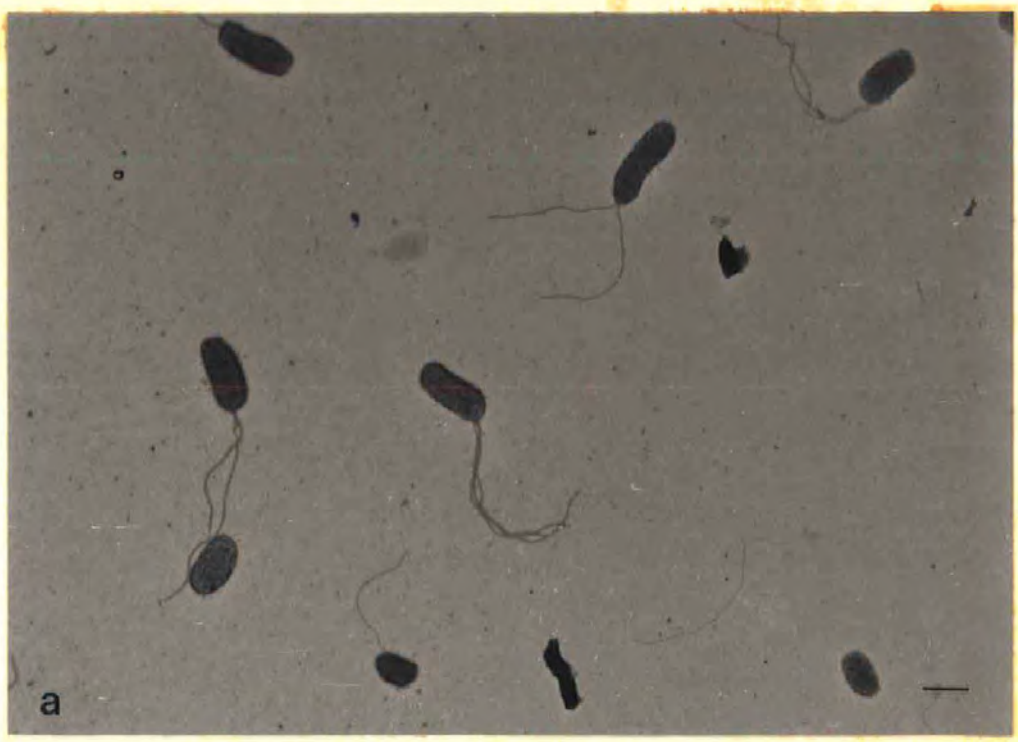
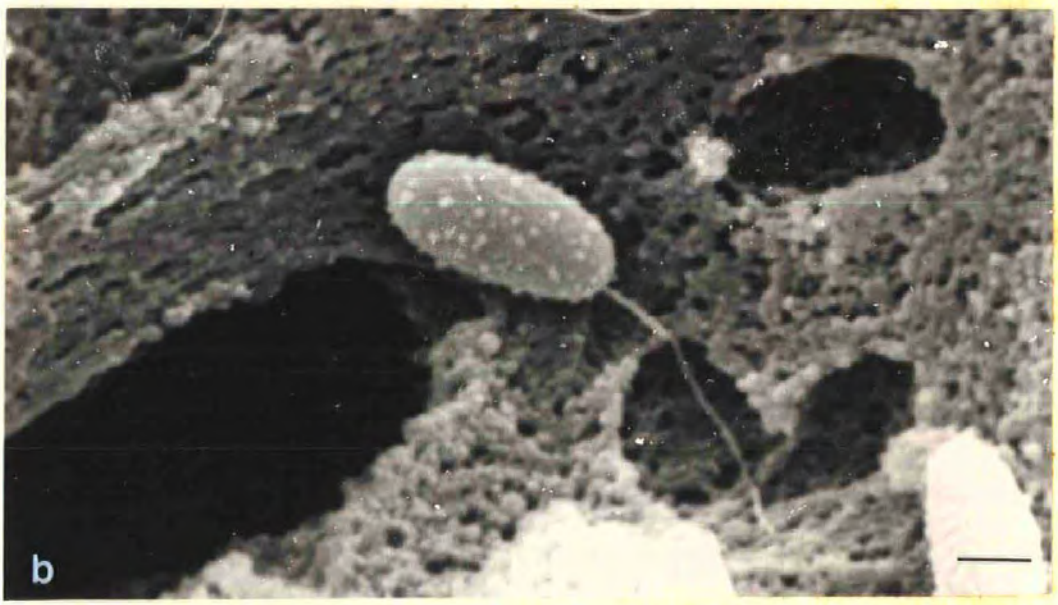

b

Plate 38 a) Electron micrograph of negative stained V.anguillarum strain HC 2 demonstrating abundance of multiflagellated bacteria (x 6000,bar=0.0.1μm).

Plate 38 b) Scanning electron micrograph of 'vibrio-like' bacterium in myocardium of infected eel demonstrating rugose appearance of cell(x 22222,bar=0.45μm).

Plate 38 c) Electron micrograph of negative stained V.anguillarum from blood of infected eel(x 66000,bar=0.25μm).


a


b

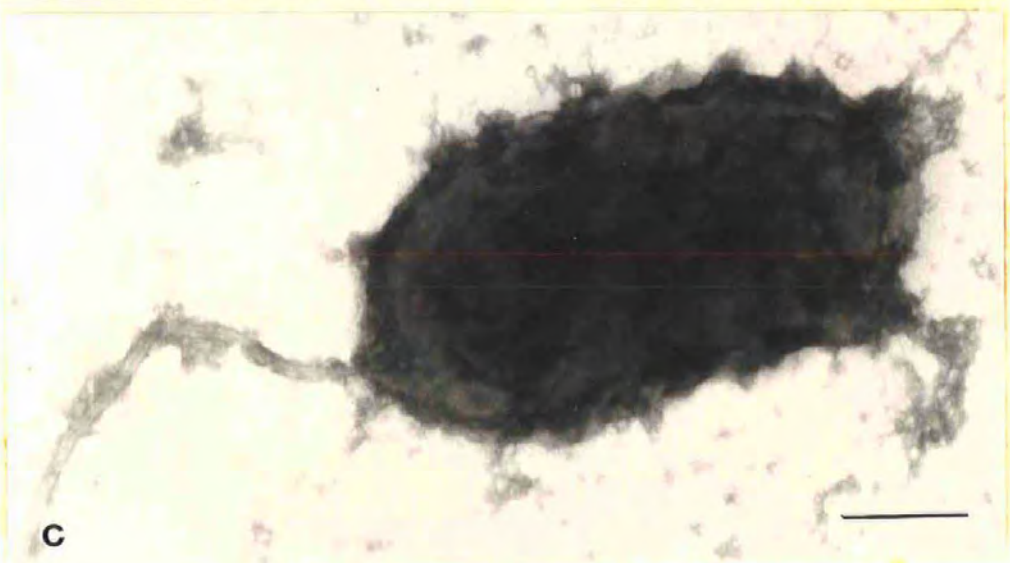
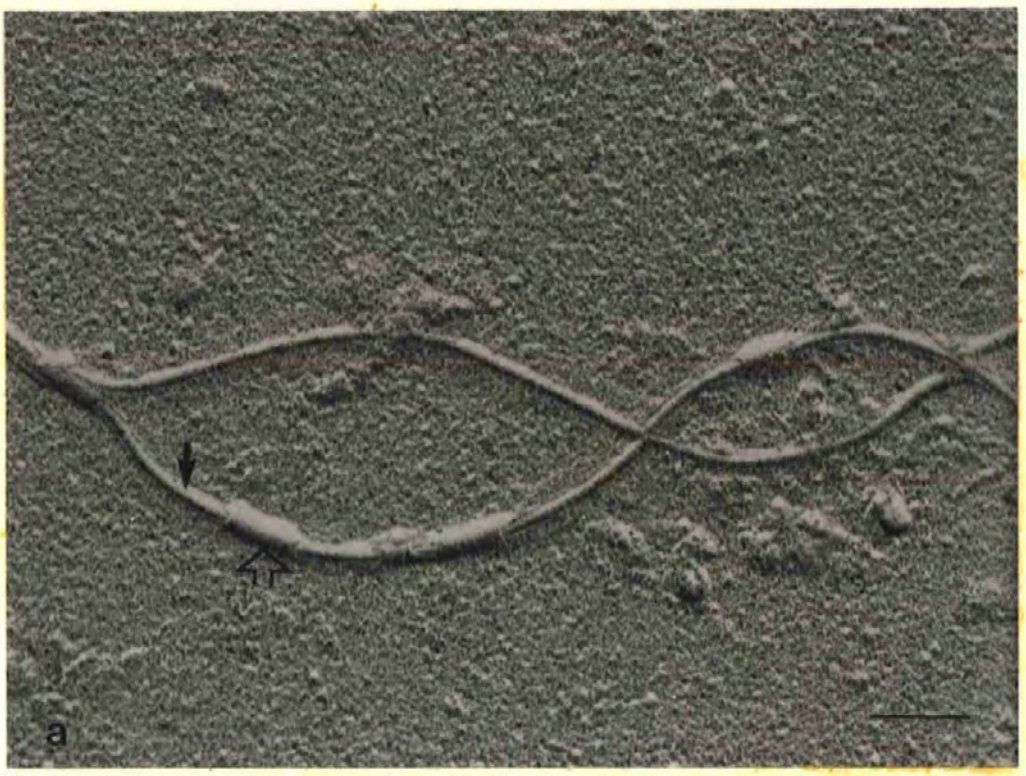

c

Plate 39 a) Electron micrograph of gold-palladium shadowed V.anguillarum flagellum showing inner core(➡) covered by outer sheath(⇨)(x 130 000,bar= 0.1µm).

f=flagella

Plate 39 b) Electron micrograph of gold-palladium shadowed V.anguillarum flagellum demonstrating terminal swelling(arrowed) (x 440 000.bar=0.05µm).

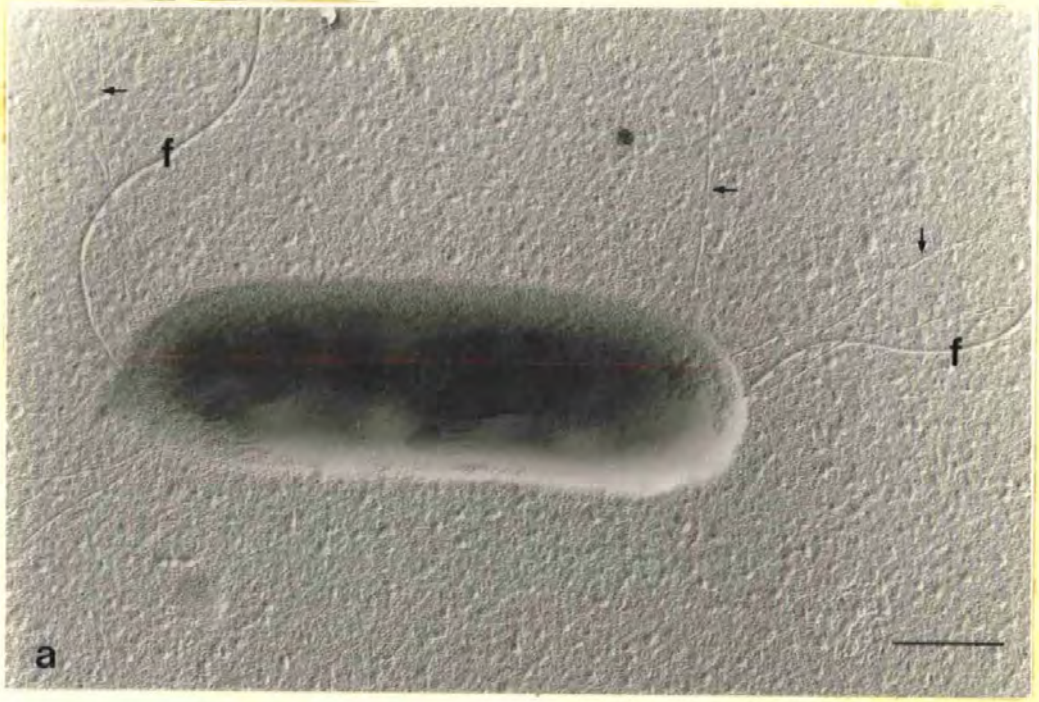
f=flagellum.


a


f
b

Plate 40 a) Electron micrograph of gold-palladium shadowed V.anguillarum demonstrating flagella(f) and pili(arrowed) (x 32500,bar=0.5μm).

Plate 40 b) Electron micrograph of negative stained V.anguillarum demonstrating flagellum(f) and pili(arrowed)(x 39000,bar=0.25μm).


f
f
a

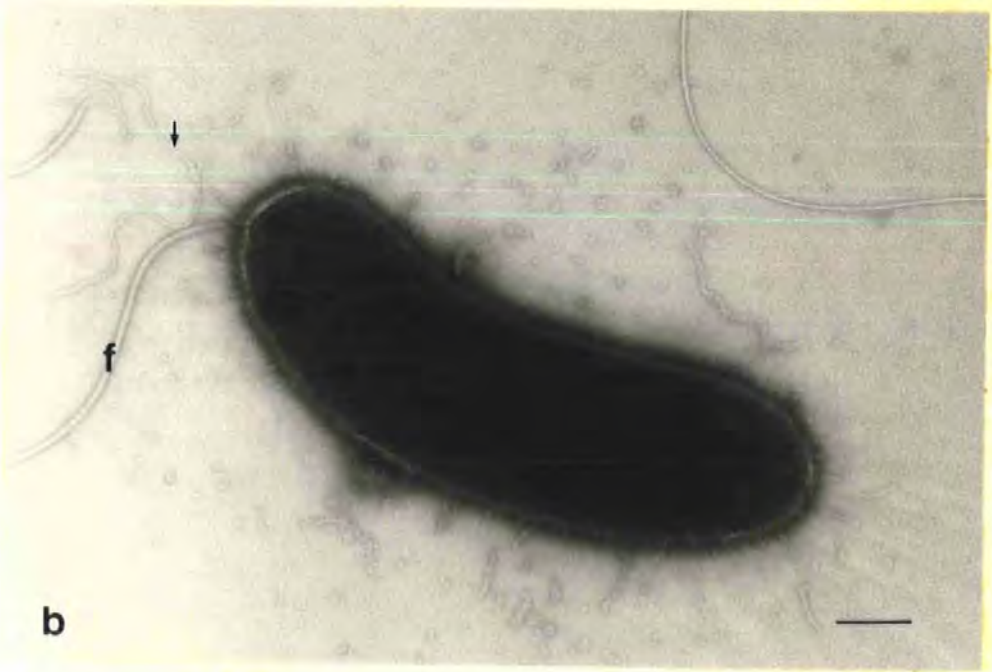
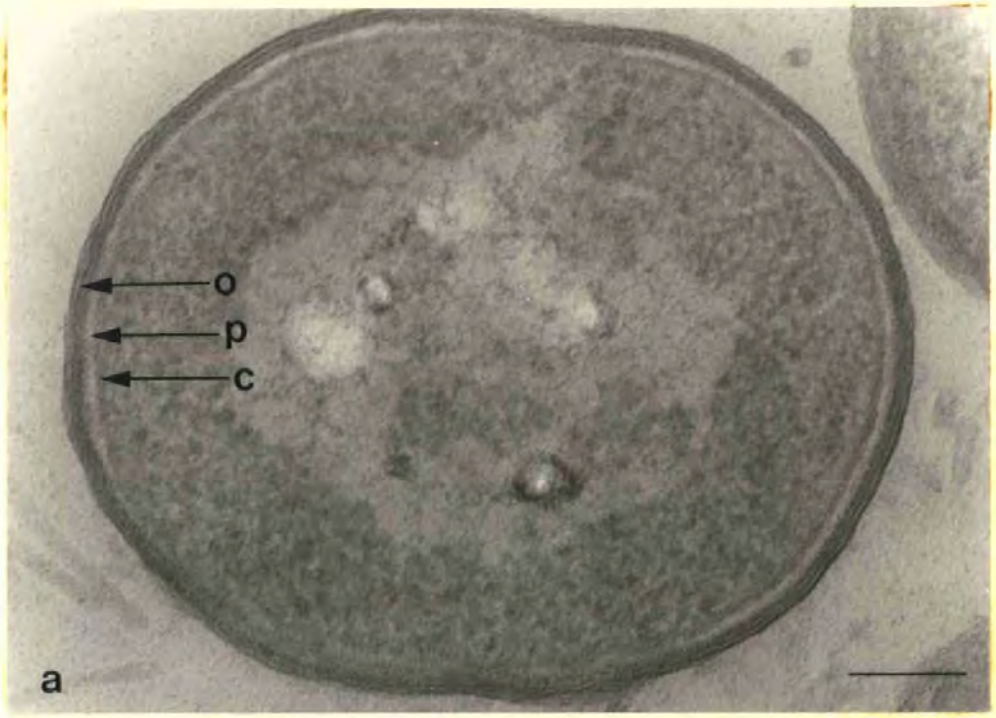

f
b

Plate 41 Electron micrograph of gold-palladium shadowed *V.anguillarum* demonstrating intracellular 'humps'(x 28000,bar=0.5μm).

Plate 42 a) Electron micrograph of agar cultured V.anguillarum cell envelope indicating outer membrane(o),periplasmic space(p) and cytoplasmic membrane(c)(x 160 000,bar=0.1µm).

Plate 42 b) Electron micrograph of'vibrio-like' bacteria in infected eel liver showing rugose outer membrane, note similar envelope topography as agar cultured bacteria (x 54000,bar=0.25µm).


o
p
c
a


ht
b

Plate 43 a) Protein profile of V.anguillarum envelope proteins extracted by guanidine-hydrochloride.

Track 1= PB-15

Track 2= MS 2072

Track 3= COB 408

Track 4= 3022A

Track 5= MS 549

Track 6= UNH 569-parent strain

Track 7= UNH 569-subcultured derivative

S= Standards

Plate 43 b) Protein profile of V.anguillarum sphaeroplasts from broth cultured(TSB) bacteria.

Track 1= PB-15*

Track 2= MS 2072*

Track 3= COB 408*

Track 4= HC 2

Track 5= 3022A

Track 6= MS 549

Track 7= UNH 569-subcultured derivative

Track 8= Unh 569-parent strain

S= Standards

Country of origin:

J=Japan

U=USA

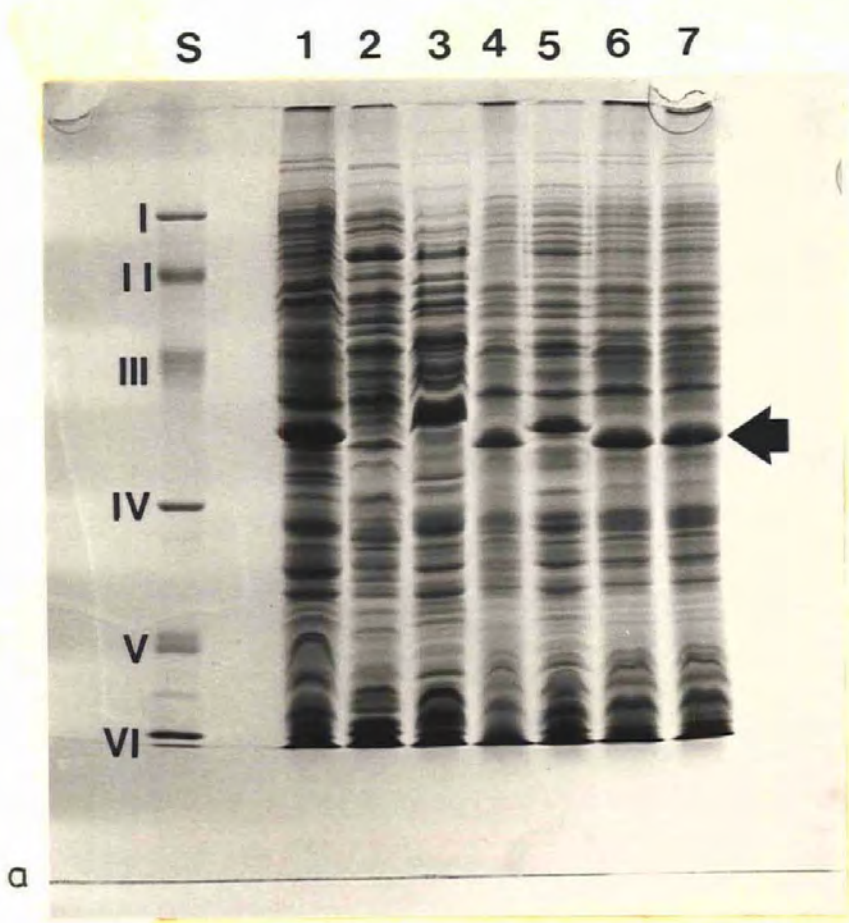
F=France

D=Denmark

*=Indole +ve

Standards:-I=phosphorylase b (94000),II=albumen(67000),III=ovalbumen(43000),IV=carbonic anhydrase(30000), V=trypsin inhibitor(20100),VI=lactalbumen(14400).

Arrow = major outer membrane protein.


S 1 2 3 4 5 6 7
I
II
III
IV
V
VI
a

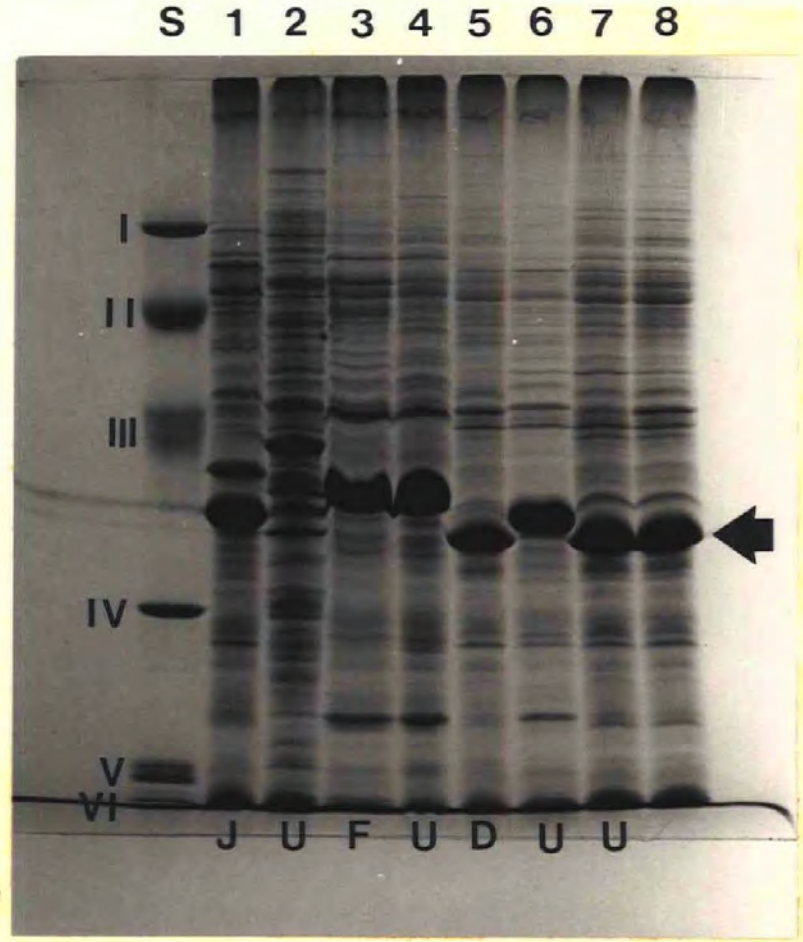

S 1 2 3 4 5 6 7 8
I
II
III
IV
V
VI
J U F U D U U
b

Plate 44 a) Protein profile of V.anguillarum

sphaeroplasts from agar cultured(TSA) bacteria, (10µl).

Track 1= UNH 569-parent strain

Track 2= UNH 569-subcultured derivative

Track 3= MS 549

Track 4= 3022A

Track 5= HC 2

Track 6= COB 408

Track 7= MS 2072

Track 8= PB-15

S= Standards

Plate 44 b) Protein profiles of V.anguillarum

sphaeroplasts from agar cultured(TSA) bacteria, (5µl).

Track 1= UNH 569-parent strain

Track 2= UNH 569-subcultured derivative

Track 3= MS 549

Track 4= 3022A

Track 5= HC 2

Track 6= COB 408

Track 7= MS 2072

Track 8= PB-15

S=Standards

Standards:-I=phosphorylase b(94000),II=albumen(67000),III=ovalbumen(43000),IV=carbonic anhydrase(30000),
V=trypsin inhibitor(20100),VI=lactalbumen(14400).

arrow = major outer membrane protein

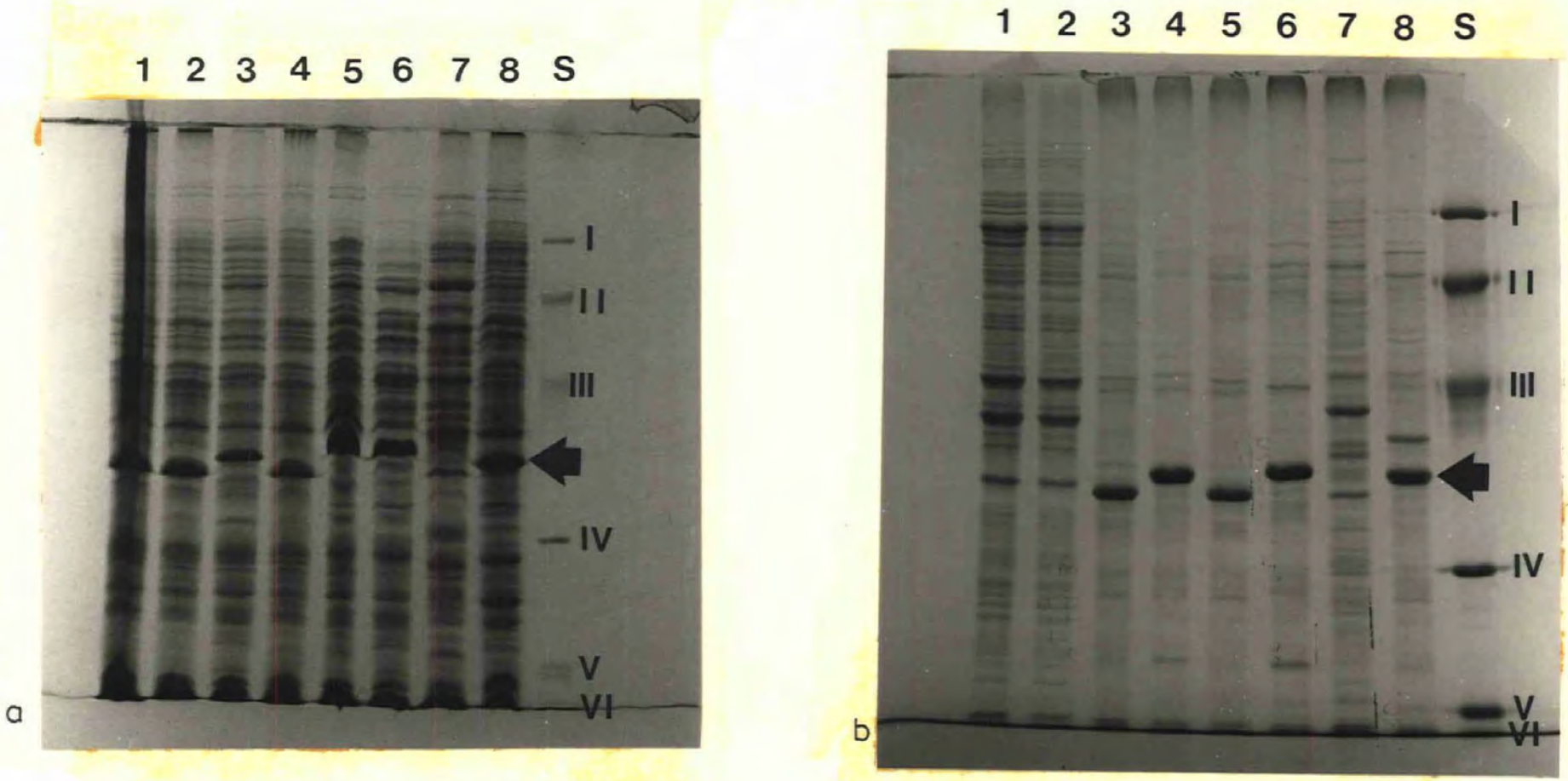

1 2 3 4 5 6 7 8 S
I
II
III
IV
V
VI
a
1 2 3 4 5 6 7 8 S
I
II
III
IV
V
VI
b

Plate 45 a) Protein profile of V.anguillarum envelope proteins extracted using 0.3M lithium acetate.

Track 1= UNH 569-parent strain

Track 2= UNH 569-subcultured derivative

Track 3= MS 549

Track 4= MS 2072

Track 5= PB-15

S= Standards.

Plate 45 b) Protein profile of V.anguillarum envelope proteins extracted by Triton X-100.

Track 1= PB-15

Track 2= MS 2072

Track 3= COB 408

Track 4= HC 2

Track 5= 3022A

Track 6= MS 549

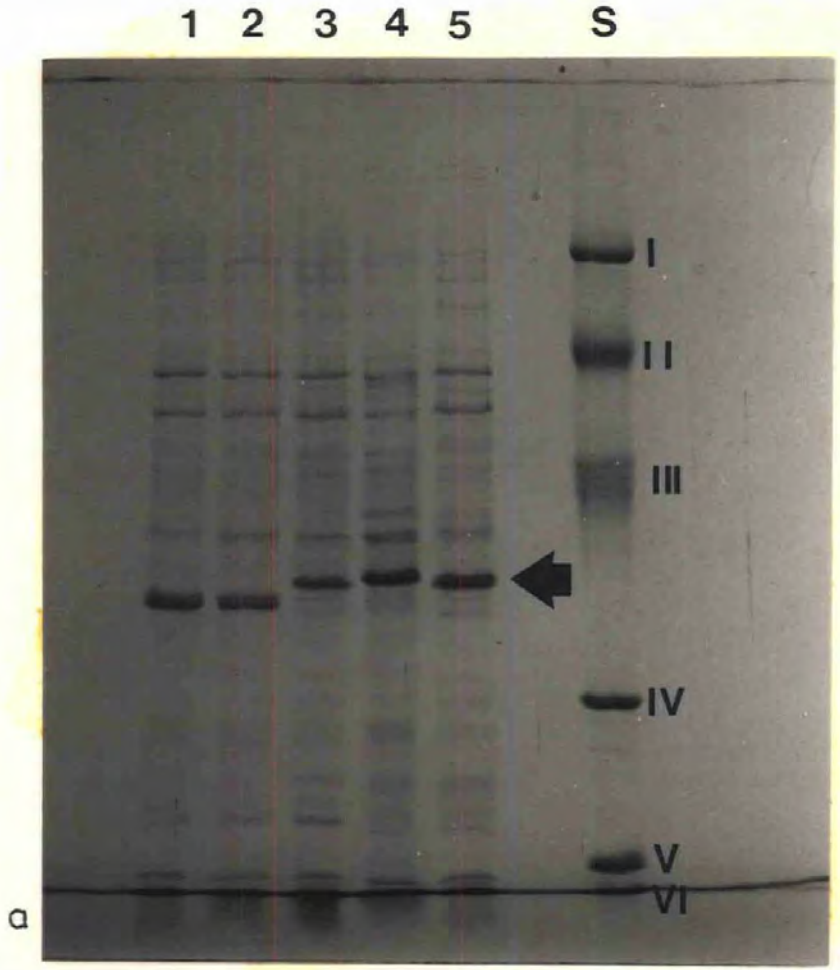
Track 7= UNH 569-subcultured derivative

Track 8= UNH 569-parent strain

S= Standards

Standards:-I=posphorylase b((94000),II= albumen(67000),III=ovalbumen(43000),IV=carbonic anhydrase(30000), V=trypsin inhibitor(20100),VI=lactalbumen(14400).

arrow = major outer membrane protein


1 2 3 4 5
S
I
II
III
IV
V
VI
a

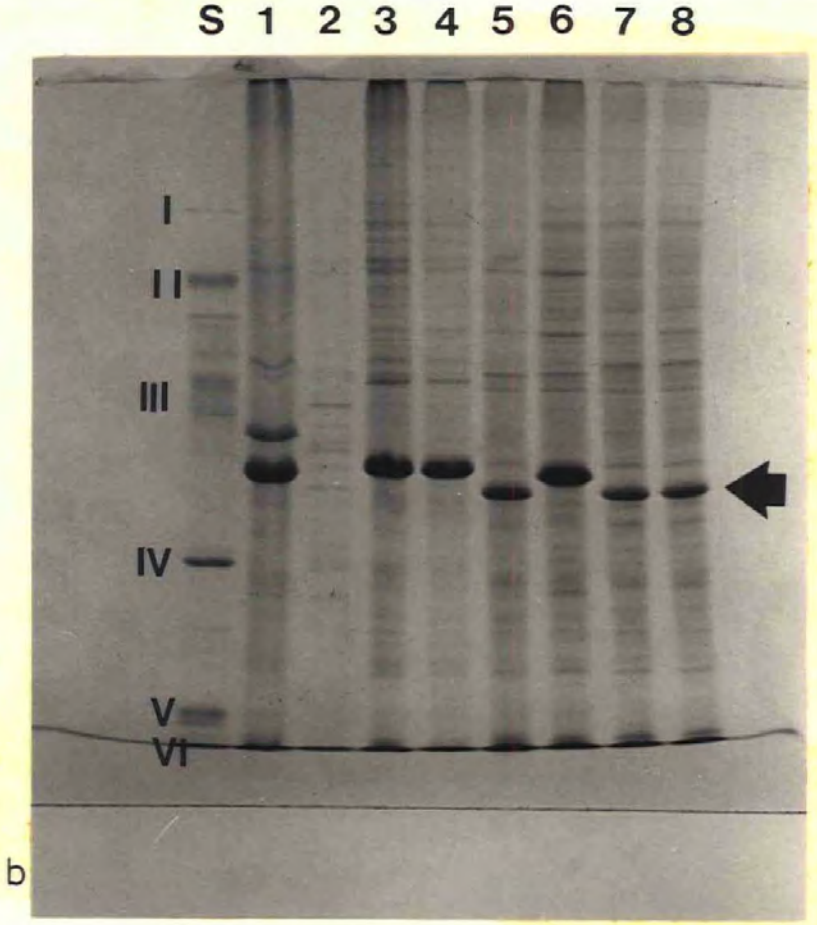

S 1 2 3 4 5 6 7 8
I
II
III
IV
V
VI
b

Plate 46 a) Protein profile of V.anguillarum strain UNH 569 envelope and outer membrane proteins from broth(TSB) cultured bacteria.

Tracks 1-3=outer membrane

Tracks 4-7=Cell envelope

S=Standards.

Plate 46 b) Protein profiles of V.anguillarum strain UNH 549 and its subcultured derivatives extracted using Triton X-100 from broth(TSB) cultured bacteria.

Tracks 1&2= UNH 569 subcultured x 20

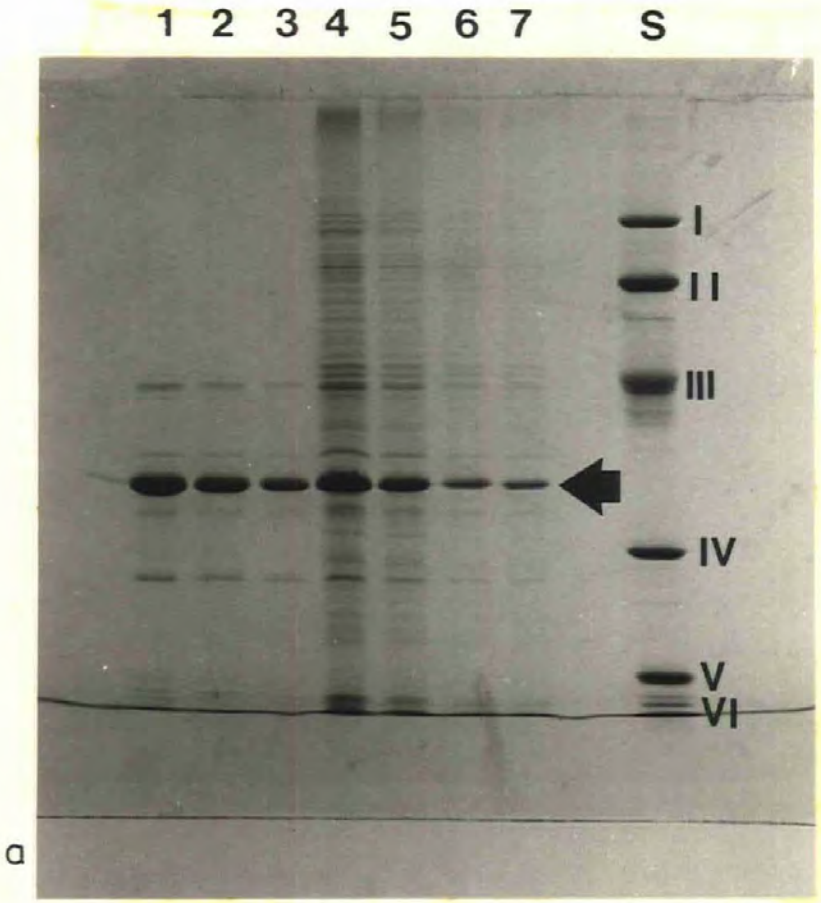
Tracks 3&4= UNH 569 subcultured x 10

Tracks 5&6= UNH 569 parent strain

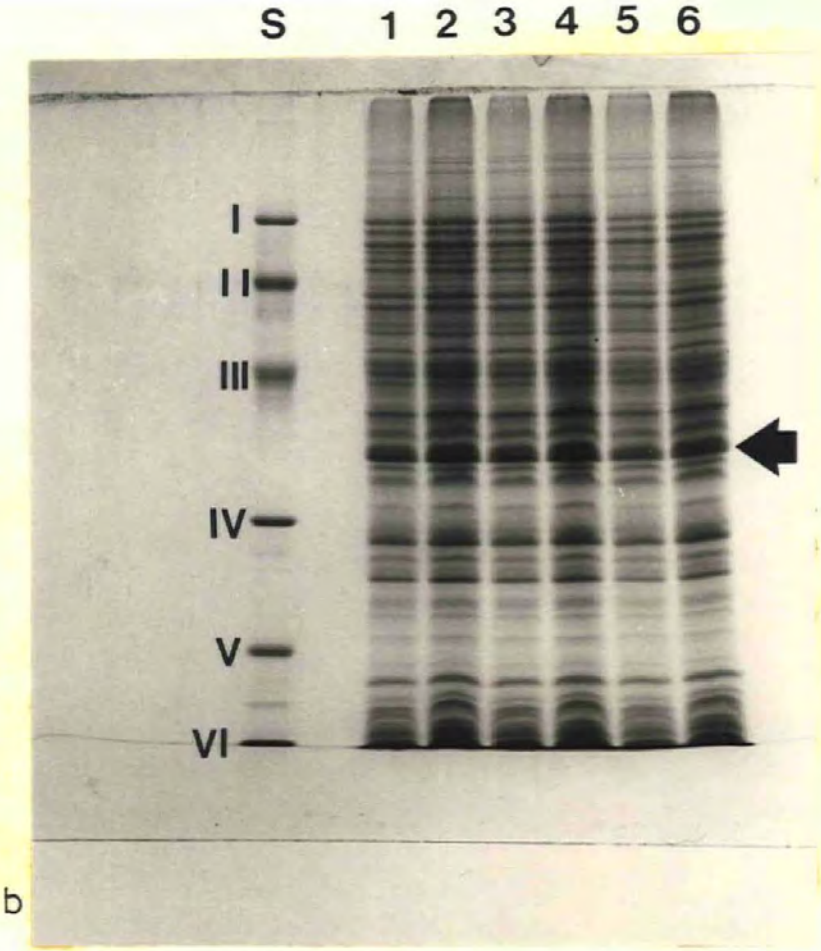
S= Standards

Standards:-I=phosphorylase b(94000),II=albumen(67000),III=ovalbumen(43000),IV=carbonic anhydrase(30000), V=trypsin inhibitor(20100),VI=lactalbumen(14400).

arrow = major outer membrane protein



a



b

Plate 47 Electron micrograph of V.anguillarum strain UNH 569 following treatment with Triton X-100,demonstrating removal of envelope material(x 60000,bar=0.25μm).

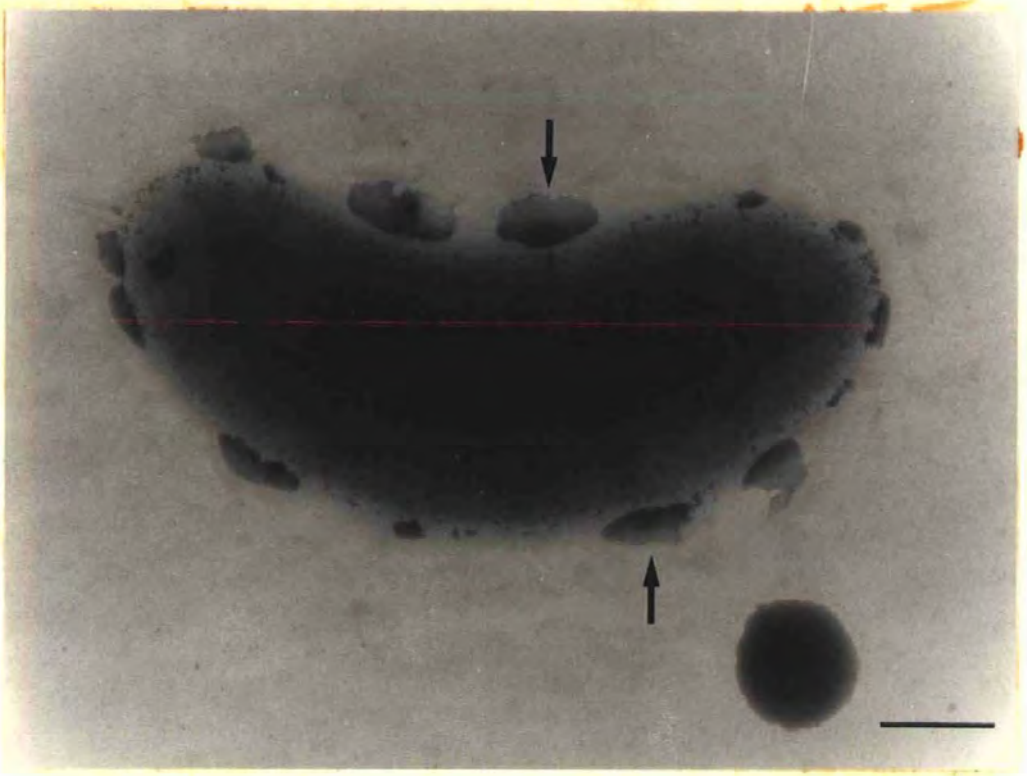


Plate 48 Crossed immuno-electrophoresis of V.anguillarum sphaeroplasts into agarose containing 'rabbit-anti-UNH 569-envelope' antisera.

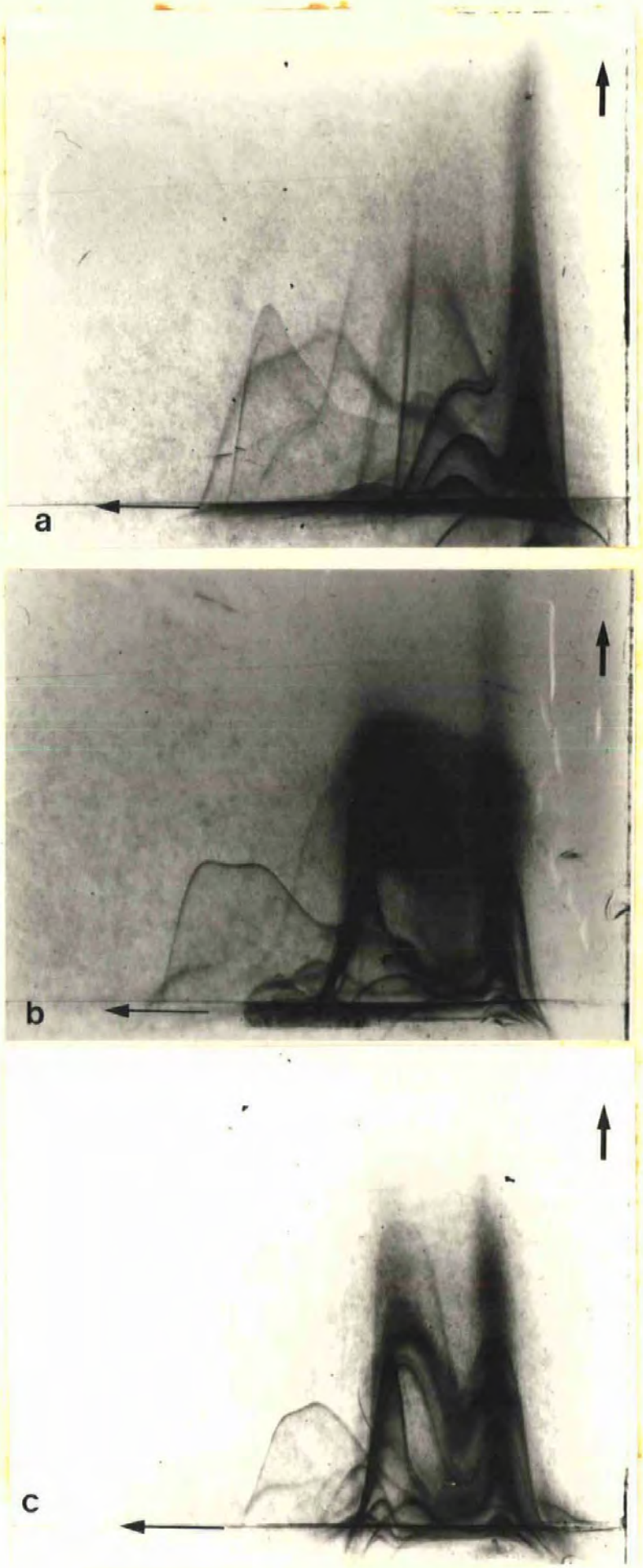
a)= UNH 569

b)= HC 2

c)= COB 408

 = 1st dimension of electrophoresis

 = 2nd dimension of electrophoresis.


a
b
c

CHAPTER 7. GENERAL DISCUSSION AND CONCLUSIONS

The present study commenced in the absence of information concerning infection of fish by *V. anguillarum*, although histopathological examination of moribund fish had been described. Apart from the work on the haemolysins of *V. anguillarum*, no attempt had been made to elucidate the pathogenicity of this economically important pathogen. In the course of the present research, there have been several contributions to this field (Harbell *et al.*, 1979; Crosa & Hodges, 1981).

The septicaemia associated with vibriosis in eels resembles that recently described in salmonids (Harbell *et al.*, 1979). The disease was characterised by large numbers of tissue bacteria prior to death, resembling, in some respects anthrax in the guinea-pig reported by Keppie *et al.* (1955), and although one cannot draw direct parallels between the two diseases, information available on the pathogenicity of *Bacillus anthracis* and it's toxins (Smith *et al.*, 1953; Keppie *et al.*, 1955; Smith & Stoner, 1967) could assist in concentrating future areas of research in vibriosis.

During the study, histopathology was monitored during pathogenesis revealing the events leading to disease and death in the eel, which has not been previously reported. This novel research demonstrated the gradual onset of disease manifestations, with the loss of gut epithelia being a fundamental feature of experimental vibriosis. Concurrent physiological studies revealed the gut desquamation to be associated with a concomitant loss of electrolytes into the intestinal lumen. In addition, this was investigated for the first time using electron microscopy, which gave valuable insight into the disease at the cellular level; in particular the demonstration of an electron translucent zone around these bacteria, thought to be as a consequence

of localised tissue damage.

The present study presents pioneer work concerning what is thought to be a likely means by which V. anguillarum enters the fish. The entry of V. anguillarum into fish tissues appears to have been a matter for conjecture.

Natural phenomena such as osmoregulation and feeding appear to give potentially pathogenic bacteria access to the fish gut, in which V. anguillarum appears particularly well adapted to survive. This organism has been shown to be able to leave the gut and survive in host tissues, particularly the kidney. Stress caused by external influences such as high temperature and/or low levels of dissolved oxygen could influence an entire fish population and could upset a fish-pathogen equilibrium in favour of the pathogen. This would explain the mass mortalities which occur during epizootics of vibriosis. In geographical locations where vibriosis is prevalent, V. anguillarum is readily isolated from the environment (Håstein, 1973; Grischkowsky, 1973; Simidu et al., 1973); failure to isolate this organism from local fish, suggesting an overall scarcity of this species, may explain the infrequent occurrence of vibriosis in local waters.

The monitoring of disease using electrocardiography has not been encountered for vibriosis or any other fish disease. The heart appeared to be particularly affected during pathogenesis with cardiac function severely impaired, probably due to bacterial invasion of the myocardium and possible effects of bacterial products. In eels obtained from a natural outbreak, the heart and particularly the atrium was severely damaged. Impaired oxygen carrying capacity of the blood due to haemolysis in association with reduced heart function would explain the increased respiration rate observed in dying fish and is probably one of the major causes of death.

This thesis presents the first detailed study of the pathogenicity of V. anguillarum and the elucidation of possible virulence factors. Most strains examined were of low virulence for eels and LD 50 values were much higher than those reported for salmonids. Examination of strains has revealed that possibly two haemolysins are produced. Haemolysin production probably occurs during pathogenesis and may be responsible for the reduction of circulatory erythrocytes reported elsewhere.

The roles of other aggressins remain unclear. Envelope proteins of V. anguillarum had not been previously investigated when the research commenced. The present study has demonstrated the V. anguillarum envelope proteins following various protein extraction techniques. The work has shown protein profiles to have a degree of strain specificity which was not noticeably altered by subculture. Most strains had a major outer membrane protein believed to be a porin. Further membrane studies should be carried out using enhanced and attenuated derivatives of parent strains, to determine whether altered surface properties are key determinants of virulence.

The rapid haemosiderin deposition in eel liver cells following injection and external challenge with V. anguillarum has not been described before. This feature was of particular interest for two reasons. Firstly, it would help to explain the role of the iron-sequestering system demonstrated by Crosa and his coworkers since strains of V. anguillarum would have to obtain this bound iron prior to multiplication in the host. The haemolysin might also be involved in iron acquisition and it's role in this requires further research. Secondly, the host defence system appears to be slow and temperature dependent hence, rapid binding of circulatory iron in contrast would appear to be an efficient and poorly studied host defence mechanism.

In addition to the present study it would be useful to examine the fate of V. anguillarum in fish infected by more natural means, as possible virulence factors involved in attachment and gut commensal competition, are over-ridden when fish are inject with inocula. Furthermore it was not known if the experimental protocol employed was optimum for virulence induction. Meynell (1961) emphasised the importance of medium, growth phase and other factors in the virulence of pathogenic bacterial features which were not fully investigated here. Although strains have characteristic features in vivo, to what extent aggressins are induced in vivo still remains unclear. It would be especially useful to study the properties of the bacterium in vivo although considerable technical problems must be overcome.

In addition, it would be of considerable interest to study V. anguillarum with respect to attachment, penetration and lysis of cells, using tissue culture; particularly with the establishment of cell lines of freshwater fish (Wolf & Quimby, 1962; Wolf, 1965; Gravel & Malsberger, 1965), marine fish (Clem et al., 1961) and in particular eel cells (Wakabyashi & Egusa, 1969).

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Experimental Vibriosis in the Eel (Anguilla anguilla)

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Introduction

Vibriosis is probably the most important disease of marine and, to a less extent, freshwater fishes. With an increase in fish aquaculture the disease has become a severe economic problem.

The organism was first mentioned by Canestrini in 1893 [5], who named the bacterium responsible for an epizootic in eels: *Bacillus anguillarum*, later to be renamed *Vibrio anguillarum* by Bergman in 1909 [3]. In eels the first external symptoms consisted of reddening of the tail and fins, and inactivity. Post-mortem examination revealed congested intestinal blood vessels, liver, and genitalia [4]. Bergman [3] reported the presence of bloody lesions in the musculature of infected fish. Literature on non-anguillid fish is too extensive to outline in this paper, however one particular feature of the disease is the presence of fluid in the alimentary canal variously described as a bloody discharge from the vent [15], clear viscous fluid in the gut [2, 11] and mucus in the gut [20]. Although symptoms of vibriosis in fish are well described, mechanisms of pathogenicity of *V. anguillarum* and the events leading to the invasion and death of the fish host appear to be unknown. Several theories for the route of entry of the pathogen have been put forward, the main ones being as a contaminant in fish-based diets [19] or via contaminated water [18, 19].

The aims of this present investigation were to determine the effects of administering inocula of *V. anguillarum* via different routes into the freshwater eel *(Anguilla anguilla)*, and the nature of a mucoid, anal exudate encountered during the experiment.

Material and Methods

Inoculation of Fish

Strains of *V. anguillarum* were obtained from various sources isolated from naturally infected fish. For virulence testing, inocula of 0.1 cm^3 (10^7 organisms) were injected intraperitoneally (IP) into groups of eels (50–70 g wt) kept in 50% sea water. Fish were also inoculated with virulent strains by intramuscular (IM) or intravascular (IV) injection, or by insertion into either the foregut or rectum by means of a catheter. Control fish received 0.1 cm^3 sterile Tryptone soy broth (TSB). Fish were externally

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challenged by placing them in 50% sea water containing approximately 10^6 viable organisms cm^{-3}. This was carried out on both intact fish and eels with a 1-cm lesion in their flank.

Culture of Organisms

Test strains were cultured in TSB [(Oxoid) 1.5% NaCl, 25 °C, 24 h]. Immediately post mortem, blood, spleen and anal exudate samples were streaked onto blood agar plates. Resulting colonies were cultured in TSB; motile, gram-negative, curved rod-shaped bacteria which were fermentative, oxidase-positive and sensitive to the vibriostatic compound 0/129 (2,4 diamino-6,7 diisopropyl pteridine phosphate) were considered *Vibrio* species. To confirm Koch's postulates, eels were injected with these isolates.

Histology

Tissues to be examined by light microscopy were fixed in 10% Formol-saline for two days. After decalcification in R.D.C. (Bethlehem Instruments Ltd.), material was dehydrated in graded alcohols, cleared in xylene and embedded in paraffin wax. Sections (10 μm) were cut using a rotary microtome, and stained with Mallory's triple stain and periodic acid Schiff's stain (PAS) for mucopolysaccharides [17].

Tissues to be examined by transmission electron microscopy (Phillips 300 TEM) were fixed in 3% gluteraldehyde in saline, post-fixed in 1% osmium, dehydrated in graded alcohols and treated with propylene oxide prior to resin embedding. Ultrathin sections cut on a Porter-Blum Ultramicrotome were stained with uranyl acetate and Reynold's lead citrate. Materials examined by scanning electron microscopy (Jeol 35C SEM) were fixed in 3% gluteraldehyde in saline (1 week) and osmicated (2 days). After dehydration the tissues were critical-point-dried (Sam Dry PVT 3) and gold-coated.

Results

Effects of Intraperitoneal Injection

From a total of 15 test strains injected IP, 7 were avirulent and 8 virulent. Virulent strains were designated as those causing death within 2 days. In most cases, the symptoms consisted of severe haemorrhaging, ulceration and liquefaction of the musculature at the site of injection. Petechial haemorrhages were present on the ventral surfaces, including the lower jaw. Congestion was prevalent in the fins and the gills were haemorrhagic. Internally, petechial haemorrhages were abundant on the wall of the peritoneal cavity and the entire alimentary canal was haemorrhagic with enlarged, congested blood vessels. The kidney, spleen and liver were haemorrhagic in most cases but the heart appeared unaffected. In most cases, anal exudate was present, but not all had fluid in the gastric caecum.

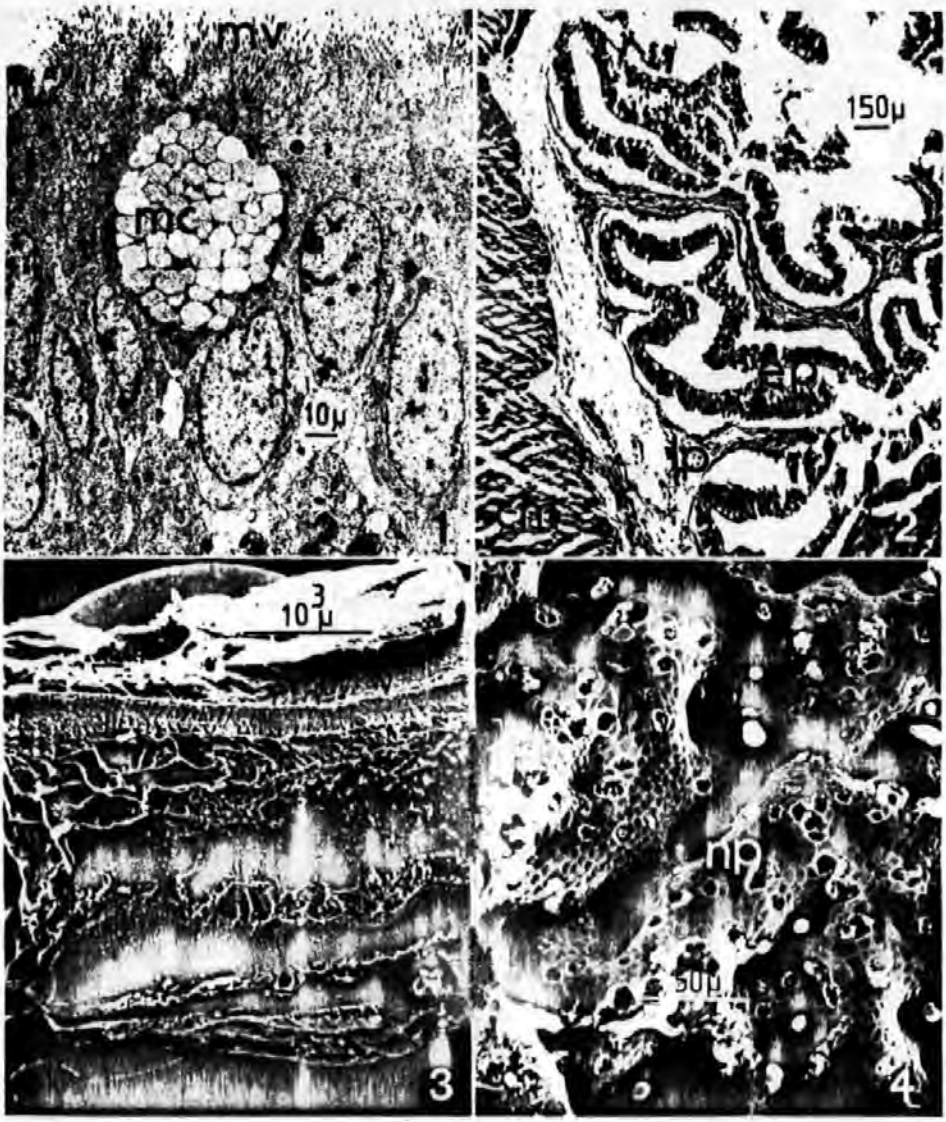

Fig. 1. Electron micrograph of gut epithelium from anal exudate *mv* microvilli; *mc* mucus cell

Fig. 2. Light micrograph of infected gut (Mallory's triple stain) *cm* circular muscle; *lp* lamina propria; *ep* epithelium

Fig. 3. Scanning electron micrograph of gut

Fig. 4. Scanning electron micrograph of gastric caecum *Np* Necrotic pits

inocula by this route. It was very surprising that fish with or without lesions, exposed to water containing vibrios, did not contract vibriosis in the three-week period, since vibrios have been found to invade host tissues by means of dermal lesions inflicted by tag labelling [18] and sea louse attachment [10].

It was evident that, regardless of inoculation route, in most diseased fish the gut epithelium had broken down, constituting a large proportion of the anal exudate. Unfortunately, there is very little information available on the effects of disease on fish tissues. Consequently, data published on mammalian pathology had to be consulted to draw parallels between situations occurring in fish and known to occur in mammals. It appears that in this case a type of inflammation is taking place. The frequently observed vasodilation of normally minute gut vessels appears to be analogous to arterial hyperaemia which takes place during inflammation. Inflammation is known to occur in fish and is closely comparable to that of mammals [8]. The irreversible cell necrosis occurring in the gut of fish with vibriosis has strong resemblance to suppuration and catarrhal inflammation exhibited in mammalian pathology [12]. Although it seems highly unlikely that normal host tissue would be affected detrimentally by its own leukocytes [12], it is possible that the presence of *V. anguillarum* or the products of necrosis caused by the action of toxins on host tissues could act as chemotactic agents for leukocytes. There have been several reports of *V. anguillarum* having gelatinase enzymes [9, 14, 16] which may play a part in the lysing of host tissues. *V. anguillarum* produces a haemolysin [13] which may be cytolytic to cells other than erythrocytes [1].

The involvement of leukocytes in the epithelial necrosis is not clear but, in the anal exudate debris, cells which may be leukocytes were present. Unfortunately, no literature on eel leukocytes has been encountered and there seems to be no clear correlation with leukocytes of other fish species [6, 7]. The steps which lead to the eventual detachment of the gut epithelium have not been investigated; however the gastric caecum from an eel that had died of the disease exhibited what would seem to be early stages in necrosis of the epithelia (Fig. 4). This focal necrosis would seem unlikely to occur in the hindgut region as sheets of epithelium are dislodged.

When studying the gut epithelia of eels the following must be considered. Firstly, eels are known to alter the structure of the gut during metamorphosis from the yellow to silver conditions. Secondly, when eels are transferred to different salinities, adaptation to the new environment involves the oesophageal epithelium [22]. The effect this has on the remainder of the alimentary canal is not known.

The reasons for the phenomena outlined in this paper and their importance in the ultimate death of fish are not clear, and further investigation is in progress.

Acknowledgement. H.C. would like to thank the SRC for the post-graduate studentship under which this work was carried out. These experiments were licenced by Home Office Licence No. SWI 2789.

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